CHARACTERIZATION OF A PEPTIDE INHIBITOR OF JANUS KINASE 2 THAT MIMICS SUPPRESSOR OF CYTOKINE SIGNALING 1 FUNCTION

By

LAWRENCE O. FLOWERS

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

ACKNOWLEDGMENTS

I am extremely grateful to my research advisor, Dr. Howard M. Johnson, for giving me the unique opportunity to train in his laboratory. His superb mentoring, motivation, and guidance have made an invaluable contribution to my life. I would also like to express my gratitude to the members of my dissertation committee—Dr. Edward M. Hoffmann, Dr. Janet K. Yamamoto, Dr. Ayalew J. Mergia, and Dr. William W. Thatcher—for their expert technical assistance, critical review of the manuscript, and patience during the production of this work.

Moreover, I express my gratitude to thank Dr. Prem S. Subramaniam for his expert advice, support, and helpful assistance with the experimental protocols used in the current studies. I wish to thank Dr. Mustafa G. Mujtaba for his excellent technical assistance. I also acknowledge Mr. Mohammad I. Haider for the peptides used in the current work. Special thanks are offered to Mr. Timothy H. Johnson for his assistance in ordering research equipment and supplies for my studies. Furthermore, I am thankful to Dr. C. M. Iqbal Ahmed and Ms. Morgan R. Ellis. Ms. Ellis was very instrumental in performing the binding assays presented in this study. This work was supported by the UNCF/Merck Graduate Science Research Dissertation Fellowship grant. The author is deeply indebted to Dr. Bhavna Bhardwaj and Mr. Neal A. Benson at the UF-ICBR Flow Cytometry Core Laboratory for technical support during the flow cytometric experiments. The support of fellow graduate students in the Microbiology and Cell Science Department is also gratefully acknowledged.

Additionally, I acknowledge various members of the faculty and support staff in the Microbiology and Cell Science Department. I would also like to thank the many nameless mentors that have guided me throughout my educational journey from elementary school to graduate school for their friendship, insightful advice, and guidance throughout my entire educational career. Lastly, I wish to thank my family and special friends for their never-ending encouragement and tireless support throughout my life and educational endeavors.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT ix

CHAPTER

1 INTRODUCTION 1

Protein Tyrosine Kinases 1
Cytokines 2
Interferon Gamma 4
Interleukin-6 7
The JAK-STAT Pathway 9
Janus Kinase (JAK) 12
Signal Transducers and Activators of Transcription 15
Negative Regulation of the JAK-STAT Pathway 18
Suppressors of Cytokine Signaling (SOCS) Protein Family 21
JAK-STAT Pathway and Cancer 26
Prostate Cancer 29
Targeted Approach to Cancer Therapy 31
Significance of the Study 33

2 MATERIALS AND METHODS 35

Peptides 35
Cell Culture and Viruses 35
Binding Assay 36
Competition Experiments 37
In vitro Kinase Assays 38
Antiproliferation Assay 40
Immunoblot Analysis 40
Flow Cytometry 42
Antiviral Assay 43
Immunoprecipitation 44
Cell Cycle Analysis 45

3 RESULTS 47

Tyrosine Kinase Inhibitor Peptide (Tkip) 47
Tkip Binds to the Autophosphorylation Site of JAK2 48
Tkip Inhibits Kinase Activity of JAK2 50
Tkip does not Inhibit Kinase Activity of VEGFR or c-Src 51
Tkip Inhibits Kinase Activity of EGFR 51
Tkip Inhibits Kinase Activity of JAK2 and EGFR in a Dose Dependent Manner 55
Tkip Preferentially Binds to the Activated JAK2 Autophosphorylation Site 55
Tkip Inhibits STAT1α Activation Not Activation of VEGFR 56
Tkip Inhibits Antiviral Activity 60
Tkip Inhibits MHC Class I Upregulation 60
Effects of Tkip on STAT3 Activation in Human Prostate Cancer Cell Lines 63
Tkip Inhibits Cell Cycle Progression in Human Prostate Cancer Cell Lines 63
Tkip Inhibits Antiproliferative Activity of PC-3 Prostate Cancer Cells 64

4 DISCUSSION 68

REFERENCES 74

BIOGRAPHICAL SKETCH 83

LIST OF TABLES

Table		page
1	Host Defense Proteins Induced by Interferon gamma	7
2	Cytokines and the JAK-STAT Pathway	10
3	Sequence Comparison of the Mammalian JAK2 Autophosphorylation Site	14
4	Sequence Comparison of the JAK Family Autophosphorylation Site	15
5	Amino Acid Sequences of the Peptides Used in this Study	48
6	Effects of Tkip on the Cell Cycle of DU145 Prostate Cancer Cells	66
7	Effects of Tkip on the Cell Cycle of LNCaP Prostate Cancer Cells	66
8	Inhibition of PC-3 Cell Proliferation by Tkip	67

LIST OF FIGURES

Figure page

1 Overview of the IFNγ Signaling Mechanism Through the JAK-STAT Pathway.....11

2 General Domain Schematic of the Janus Kinase (JAK) Protein Family.....15

3 General Domain Schematic of the Signal Transducer and Activator of Transcription (STAT) Protein Family.....18

4 General Domain Schematic of the Suppressor of Cytokine Signaling (SOCS) Protein Family.....23

5 Binding of Tkip by JAK2 Autophosphorylation Peptide, JAK2WT 49

6 Kinase Inhibitory Specificity of Tkip.....50

7 Tkip Does Not Inhibit Autophosphorylation of VEGFR 52

8 Tkip Does Not Inhibit Tyrosine Phosphorylation Activity of c-Src.....53

9 Tkip Inhibits Autophosphorylation of EGFR 54

10 Positive Control for In Vitro Kinase Assay.....54

11 Dose Response of Tkip Inhibition of JAK2, IFNGR-1, and EGFR Phosphorylation In Vitro.....56

12 Binding of Unphosphorylated JAK2 WT Peptide Versus Phosphorylated JAK2 WT Peptide to Tkip.....57

13 Tkip Inhibits IFNγ Induced STAT1α Activation in WISH Cells But Does Not Inhibit VEGF Induced Activation of VEGFR in Bovine Aortic Endothelial Cells (BAECs).....59

14 Tkip Inhibits the Antiviral Activity of IFNγ Against EMC Virus on WEHI-3 Cells.....61

Figure page

15 Downregulation of IFNγ Induced Cell Membrane Expression of MHC Class I on WISH Cells Using Tkip 62

16 Effects of Tkip on Human Prostate Cancer Cell Lines DU145 and LNCaP 65

17 Proposed Model of Inhibition of IFNγ Mediated Signaling by Tkip 70

18 Alignment of the KIR of SOCS-1 and Tkip 73

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF A PEPTIDE INHIBITOR
OF JANUS KINASE 2 THAT MIMICS SUPPRESSOR OF
CYTOKINE SIGNALING 1 FUNCTION

By

Lawrence O. Flowers

August 2004

Chair: Howard M. Johnson
Major Department: Microbiology and Cell Science

Tyrosine kinases are extremely important biological molecules involved in numerous cellular signal transduction events. Fidelity of these signal transduction events is essential for normal cellular functions. Many diseased states such as cancer and autoimmune diseases are associated with defective tyrosine kinases. Positive and negative regulation of cytokines such as gamma interferon (IFNγ) is key to normal homeostatic function. Negative regulation of IFNγ in cells occurs via proteins called suppressors of cytokine signaling (SOCS) 1 and 3. SOCS-1 inhibits IFNγ function by binding to the autophosphorylation site of the tyrosine kinase JAK2.

A short 12-mer peptide, WLVFFVIFYFFR (Tkip), was developed that bound to the autophosphorylation site of JAK2, resulting in inhibition of its autophosphorylation as well as its phosphorylation of IFNγ receptor subunit IFNGR-1 tyrosine 440. The JAK2 tyrosine kinase inhibitor peptide (Tkip) did not bind to or inhibit tyrosine

autophosphorylation of vascular endothelial growth factor receptor (VEGFR) or phosphorylation of a substrate peptide by the protooncogene tyrosine kinase c-src. Tkip also inhibited epidermal growth factor receptor (EGFR) autophosphorylation, consistent with the fact that EGFR is regulated by SOCS-1 and SOCS-3, similar to JAK2. Although Tkip binds to unphosphorylated JAK2 autophosphorylation peptide, it binds significantly better to tyrosine 1007 phosphorylated JAK2 autophosphorylation peptide. SOCS-1 only recognizes the JAK2 site in its phosphorylated state. Thus, Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1, but not precisely the same way.

Consistent with inhibition of JAK2, Tkip inhibited antiviral activity and MHC class I upregulation on cells at a concentration of approximately 10 μM. This is similar to the K_d of SOCS-3 for the erythropoietin receptor. These data represent a proof-of-concept demonstration of a peptide mimetic of SOCS-1 that regulates JAK2 tyrosine kinase function.

CHAPTER 1
INTRODUCTION

Protein Tyrosine Kinases

Protein tyrosine kinases are enzymes that catalyze the transfer of terminal phosphate groups from ATP (gamma-phosphoryl group) to a specific substrate tyrosine residue (hydroxyl group). There are approximately 90 human protein tyrosine kinases in the human genome. Two major types of protein tyrosine kinases exist in nature: receptor protein tyrosine kinases and nonreceptor protein tyrosine kinases. Receptor protein tyrosine kinases (EGFR, VEGFR) are membrane proteins that have an intrinsic kinase domain that is activated upon extracellular ligand binding to the receptor. Nonreceptor protein tyrosine kinases (JAK, Src) are soluble cytosolic proteins that mediate various signal transduction pathways (Robinson et al., 2000).

Protein tyrosine kinases can also be grouped as either an autophosphorylation protein tyrosine kinase (JAK2) or a nonautophosphorylation protein tyrosine kinase (c-Src). Autophosphorylation protein tyrosine kinases phosphorylate their own tyrosine residues and phosphorylate tyrosine residues on other proteins. Those tyrosine kinases that phosphorylate their own tyrosine residues phosphorylate amino acid residues in a specific autophosphorylation site. Nonautophosphorylation protein tyrosine kinases only phosphorylate tyrosine residues on other proteins. Protein tyrosine kinases represent a major class of kinases in eukaryotic organisms. Protein tyrosine kinases are involved in many essential cellular functions including proliferation, differentiation, cell activation,

transcription, and apoptosis. These processes work together, via complex intracellular mechanisms, to maintain homeostasis. Protein tyrosine kinases become activated by protein phosphorylation and typically become deactivated by dephosphorylation events. Ligand binding induces protein tyrosine kinase phosphorylation (activation) of the receptor or soluble intracellular nonreceptor protein. This activation leads to phosphorylation of other molecules in the signal transduction pathway. Phosphorylation of downstream components in the pathway either creates docking sites or serves to activate necessary signaling proteins. The interaction with a complex network of molecules results in the transmission of appropriate signals throughout the cell to induce specific biological effects in response to cellular stimulation (Schlessinger, 2003).

Mutations in protein tyrosine kinases often lead to abnormal cell function and carcinogenesis. Defective protein tyrosine kinases are common etiological factors for a variety of carcinomas including breast, prostate, gastric, and colon cancer. Understanding the role of protein tyrosine kinases in oncogenesis and disease progression is of great importance to immunologists. Much research is directed at identifying particular tyrosine kinase markers for detection of potential oncological disorders and developing specific tyrosine kinase inhibitors that may prove useful in counteracting the cellular effects observed in many carcinomas.

Cytokines

Cytokines are important regulators of the immune system. These cellular chemical messengers are small molecular weight proteins secreted by leukocytes and other immune cells and bind to extracellular receptors on the surface of target cells to initiate signal transduction pathways (Ishihara et al., 2002). Signal transduction pathways

lead to the modulation of gene expression in the nucleus. Cytokine-specific proteins produced as a result of gene expression mediate critical biological effects in response to the stimulatory signal. Generally, cytokines exert a multitude of specific effects on the cells of the immune system such as promoting cell proliferation, cell differentiation, hematopoiesis, cell death, and initiating host defense mechanisms (Ishihara et al., 2002).

Cytokines exhibit autocrine, paracrine, or endocrine actions as a means of receptor engagement. Autocrine action occurs when a cell receptor binds a cytokine on the same cell from which the cytokine was secreted. Paracrine action occurs when a cell receptor binds a cytokine secreted from a cell in close proximity. Endocrine action occurs when a cell receptor binds a cytokine secreted from a distant region in the body. Endocrine actions are facilitated by the circulatory system for cytokine transport. Cytokines largely consist of α-helical structure and possess extremely low dissociation constants (10^{-10} to 10^{-12}) for their membrane-bound cellular receptor.

Cytokines share the properties of synergism, antagonism, redundancy, cascade induction, and pleoitropy. Synergism occurs when the actions of two or more cytokines produce a biological effect that is stronger than the effects of the individual cytokines alone. Antagonism occurs when the action of one cytokine blocks the action of another. Antagonism also can be observed at the level of the receptor. In cases where receptor components are shared (e.g., signal transducing subunits) or similar (e.g., cytokine binding receptor) antagonistic effects are observed when there is binding competition of structurally similar cytokines for a limited number of receptor components (Fitzgerald et al., 2001). Currently there are four families of cytokines: interferon (e.g., IFNγ, IFNα, IFNβ), hematopoietin (e.g., IL-2, IL-3, IL-5, IL-12), chemokine (e.g., IL-8), and tumor

necrosis factor (e.g., TNFα, TNFβ) family. The interferon or type II cytokines and hematopoietin or type I cytokines classes represent the largest classes of cytokines. The receptors for these cytokine families are grouped in one of five classes of cytokine receptors: class I, class II, tumor necrosis factor, chemokine, and immunoglobulin receptors (Fitzgerald et al., 2001).

Interferon Gamma

Interferons were discovered in 1957 by Issacs and Lindenmann, who observed that virus-infected cells produced a substance that could protect uninfected cells from viral infection. Since that landmark discovery interferons have now been classified into major families:Type I and Type II interferons.Type I interferons consist of IFNα, IFNβ, IFNω, and IFNτ, while IFNγ is the sole representative of the type II interferons. The primary biological role of type I interferons involves the inhibition of viral replication. Type II interferons also participate in antiviral processes but are involved in other key immunoregulatory processes in the mammalian system. Type I and type II interferons are further distinguished by the receptors that mediate their biological functions. Type I and type II interferons are members of the class II cytokine receptor family. Type I interferons bind to a membrane bound receptor composed of two subunits: IFNAR-1 (530 amino acids, 100 kDa) and IFNAR-2 (490 amino acids, 95 kDa). Type II interferons bind to a different type of type II cytokine receptor consisting of two membrane bound subunits, IFNGR-1 (470 amino acids, 90 kDa) and IFNGR-2 (300 amino acids, 62 kDa) (Fitzgerald et al., 2001).

One of the most widely studied cytokines is the interferon gamma (IFNγ) cytokine system (Table 1). IFNγ is a homodimeric glycoprotein with a molecular weight

of approximately 50 kDa. IFNγ is primarily secreted by T helper cells (T_H), cytotoxic T cells (T_C), and natural killer cells (NK). Target cells for this cytokine typically include macrophages, B cells, as well as other types of cells. Although the actions of IFNγ are varied, they typically involve the promotion of an antiviral and antiproliferative state, antitumor activity, upregulation of class I and class II major histocompatibility (MHC) molecules, and the induction/suppression of specific antibodies in B cells that promote phagocytosis (Johnson et al., 1994; Boehm et al., 1997; Stark et al., 1998). IFNγ is a signature cytokine of the immune system most notably involved in the activation, differentiation, and proliferation of a subset of T_H cells known as the T helper 1 (T_H1) cell subset. T_H1 cells are mainly responsible for cell-mediated immune functions such as inflammatory actions, phagocytosis, and delayed-type hypersensitivity responses. In addition, IFNγ serves to suppress the T helper 2 (T_H2) cell subset associated with humoral-mediated immune functions (Boehm et al., 1997; Stark et al., 1998). Like most cytokines, IFNγ must bind to its cognate receptor in order to elicit biological action. The IFNγ receptor system is a heterodimeric protein unit consisting of a larger ligand binding polypeptide chain (IFNGR-1) and a smaller signal transducing subunit (IFNGR-2). IFNGR-1 is known to be highly involved in promoting a cellular response to IFNγ. The IFNγ receptor lacks intrinsic kinase ability and therefore must rely on the presence of a family of receptor associated protein tyrosine kinases for signal transmission (Bach et al., 1997; Petska et al., 1997).

The most commonly studied IFNγ induced proteins are major histocompatibility complex proteins (MHC), nitric oxide synthase (NOS), and the proteins that mediate antiviral actions including protein kinase (PKR), oligoadenylate synthetase (OAS), and

adenosine deminase (ADAR1) (Samuel, 2001). MHC proteins are cell surface membrane molecules that are essential for antigen presentation to T lymphocytes.

The major histocompatibility complex consists of class I, class II, and class III molecules. MHC class I molecules present antigens to $CD8^+$ T cells. MHC class II molecules present antigen to $CD4^+$ T cells. MHC class III molecules include complement proteins and tumor necrosis factor proteins (TNFα/β). These molecules are critical in cell mediated and humoral immune responses. In humans the MHC is termed the human leukocyte antigen complex (HLA) and in mice the MHC is termed the H-2 complex.

Nitric oxide synthase is an IFN-inducible protein that catalyzes the conversion of L-arginine to nitric oxide (NO) and L-citrulline. NO is a powerful reactive nitrogen intermediate gas that plays a significant role in mediating antimicrobial activity (Samuels, 2001).

PKR becomes activated via phosphorylation by either double-stranded or single-stranded viral RNA molecules. Upon activation PKR phosphorylates several substrates that promote the inhibition of mRNA translation in virus infected cells (Samuels, 2001).

OAS is activated by double-stranded RNA and catalyzes the synthesis of 2'5'-oligoadenylates. RNase L, a endoribonuclease, binds to 2'5' oligoadenylates and thereby activates the endoribonuclease which mediates the degradation of viral mRNA (Samuels, 2001).

ADAR1 is an RNA-specific enzyme that promotes the posttranscriptional deaminiation of adenosine to inosine. This modification of RNA generates unstable viral transcripts that are unable to participate in translational events (Samuels, 2001).

Table 1. Host Defense Proteins Induced by Interferon gamma[a]

Protein	Function
Surface Proteins	
MHC antigens	Antigen presentation
High Affinity Fc receptor for IgG	Binding and phagocytosis of opsonized particles
Enzymes	
(2'-5') oligo A synthetase E	(2'5') $(A)_n$ synthesis with activation of ribnuclease L
Indolamine 2,3-dioxygenase	Tryptophan degradation
NADPH oxidase	Superoxide generation
Nitric oxide synthase	Nitric oxide generation
Transcription Factors	
Interferon-stimulated gene factor-3γ	Part of interferon-stimulated gene factor-3
Interferon regulatory factor-1	Necessary for expression of nitric oxide synthase
Cytokines	
TNFα	Macrophage activation, granuloma formation
Interferon γ inducible protein 10	Lymnphocyte chemotaxis
Monocyte chemotactic protein 1	Monocyte chemotaxis

*NADPH = reduced form of nicotinamide adenine dinucleotide phosphate.

[a]reviewed in Gallin et al. (1995)

Interleukin-6

Interleukin-6 (IL-6), also called B-cell stimulatory factor 2 (BSF-2, BCDF), is a pleiotropic glycoprotein that primarily targets B cells, stem cells, and liver cells. The term pleiotropic refers to a cytokine that confers different biological effects on different types of target cells (Heinrich et al., 2003). IL-6 has a molecular weight of 26 kDa containing an approximately 200 amino acid sequence and is secreted by a number of cells including monocytes, macrophages, T_H2 cells, fibroblasts, cancer cells, endothelial cells, and bone-marrow cells. Biological functions include antibody secretion of plasma cells, terminal maturation of B cells to plasma cells, hematopoietic cell growth and development, and synthesis of inflammatory proteins of liver cells (Heinrich et al., 2003). Based on the biological functions listed above it is clear that IL-6 plays a key role in the acquired immune response and in the inflammatory process. The IL-6 receptor family, similar to the interferon receptor family, does not contain intrinsic kinase domains. Instead the IL-6

receptor relies on members of the Janus kinase protein family to activate (phosphorylate) its receptor complex following ligand binding to the high affinity receptor. IL-6, IL-11, IL-12, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF) are all included in the IL-6 receptor subfamily. This receptor subfamily consists of a cytokine-specific binding subunit and a common signal transduction subunit, known as gp130 which largely accounts for the similar downstream biological functions observed from the binding of the different cytokines in this subfamily (Heinrich et al., 2003). The JAK kinase proteins thought to be involved in IL-6 signaling are JAK1 and JAK2. The main STAT protein believed to be involved in IL-6 signaling is STAT3. SOCS1 and SOCS3 have further been implicated in the inhibition of IL-6 signaling (Alexander, 2002). Therefore, IL-6 binding to the IL-6 receptor leads to the formation of the IL-6-IL-6R-gp130 receptor complex. Formation of this complex leads to the activation of gp130-associated JAK molecules and subsequent later signaling events.

Constitutive secretion of IL-6 and increased IL-6 levels are believed to be linked with prostate cancer. The effect of IL-6 on the growth of prostate cancer cells has been studied extensively recently (Culig et al., 2002; Pfitzenmaier et al., 2003; Smith et al., 2001). Lee et al. (2003) demonstrated that cell proliferation was enhanced in LNCaP prostate cancer cells overexpressing IL-6 mediated by an increased STAT3 phosphorylation. It was also determined in these studies that IL-6 augmented the growth of tumors in mice. Additionally, elevated IL-6 levels in the serum are observed in cachetic prostate cancer patients. This suggests that increased IL-6 levels may influence the development of cachexia, a wasting disease, in prostate cancer patients (Pfitzenmaier et al., 2003).

The JAK-STAT Pathway

The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is the major pathway for cytokine signaling (Table 2). Intracellular signaling events in the JAK-STAT pathway led to the observed cellular functions associated with the ligand. The Janus kinase family of nonreceptor tyrosine kinase proteins are tyrosine kinases that associate with cytokine receptor subunits. In the case of the IFNγ signaling pathway, activation involves two members of the JAK tyrosine family, JAK1 and JAK2 (Figure 1). JAK1 has been shown by immunoprecipitation studies to associate with IFNGR-1 while JAK2 has been shown to associate with IFNGR-2 in the absence of IFNγ treatment. Upon ligand binding, however, dimerization of the receptor subunits occur, bringing the two JAK molecules in close proximity and allowing for their autophosphorylation and subsequent IFNGR-1 activation. Recent studies with monomeric IFNγ show that receptor dimerization is not obligatory for subsequent signal transduction events. Once activated, JAK molecules phosphorylate the receptor subunit. JAK-mediated phosphorylation of specific residues on the receptor subunit (Tyr 440) provides unique docking sites for the STAT1α monomers, which bind to the phosphotyrosine on the receptor subunit through src homology domain 2 (SH2), a highly conserved domain located on all STAT molecules. JAK proteins then phosphorylate STAT1α at specific tyrosine residues (Tyr 701). Additional phosphorylation of STAT1α at serine residue 727 is required for transcriptional activity. Phosphorylation of STAT1α causes the molecule to be translocated to the nucleus by as yet unknown mechanisms. Following translocation to the nucleus, STAT1α binds to gamma activating sequences

(GAS) and activates transcription of IFNγ cellular response genes (Johnson et al., 1994; Samuel, 2001).

There is a great deal of cross-talk between JAK-STAT pathways mediated by several different cytokines, growth factors, and hormones (Table 2). For example, both type I and type II inteferons mediate biological activity by activating STAT1. Additionally, cross talk is observed between JAK-STAT and nuclear factor-κB signaling pathways through SOCS-1 regulation. Upon activation of both the JAK-STAT and nuclear factor-κB signaling pathways, SOCS-1 expression is induced to negatively regulate both pathways (Shuai and Liu, 2003).

Table 2. Cytokines and the JAK-STAT Pathway[a]

Cytokine	JAK	STAT
IL-2, IL-7, IL-9, IL-15	JAK1, JAK3	STAT5
IL-3, IL-5	JAK2	STAT5
IL-6	JAK1, JAK2	STAT3
IL-10	JAK1, TYK2	STAT3
IL-12	JAK2, TYK2	STAT4
IL-13	JAK2	STAT3, STAT6
Leptin	JAK2	STAT3
Erythropoietin	JAK2	STAT5
Growth Hormone	JAK2	STAT3, STAT5
EGF	JAK1, JAK2	STAT1, STAT3
IFNγ	JAK1, JAK2	STAT1
IFNα, IFNβ	JAK1, TYK2	STAT1-STAT6
IFNτ	JAK1, TYK2	STAT1, STAT2

[a]Selected cytokines listed with JAK kinases and STAT transcription factors believed to be involved in cytokine signaling. Abbreviations: JAK, Janus kinase; Tyk, tyrosine kinase; STAT, signal transducer and activator of transcription; IFN, interferon; IL, interleukin; EGF, epidermal growth factor. Adapted from Schindler, 2002; Luo and Laaja, 2004.

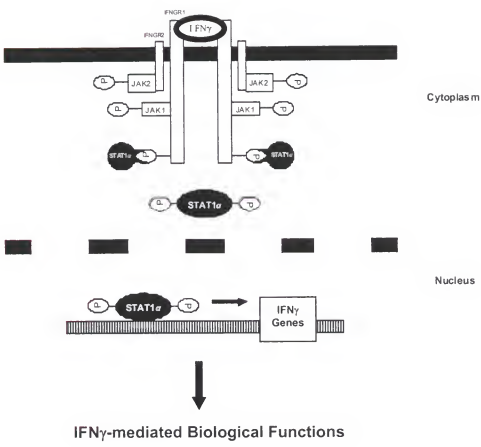


Figure 1. Overview of the IFNγ Signaling Mechanism Through the JAK-STAT Pathway. This model depicts the prevailing view of the intracellular signaling events of the JAK-STAT pathway that are initiated following cytokine stimulation. Cytokine binding promotes receptor dimerization, JAK kinase activation, receptor and STAT protein phosphorylation. Activated STAT molecules dimerize and are translocated to the nucleus to promote expression of IFNγ responsive genes.

Janus Kinase (JAK)

The mammalian family of JAK nonreceptor tyrosine kinase proteins contains four members: JAK1, JAK2, JAK3, and Tyk2. JAK proteins (>1000 amino acids) are approximately 120 to 130 kDa cytosolic proteins expressed in many types of tissue with the exception of JAK3 whose expression is restricted to natural killer cells and thymocytes (Leonard and O'Shea, 1998). Using JAK1-JAK2 chimeric proteins in $JAK1^{-/-}$ cell lines (Kohlhuber et al., 1997), it was demonstrated that the specificity of the JAK-STAT pathway is not entirely dependent on the JAK proteins and may involve other components of the JAK-STAT pathway to ensure fidelity of signal transduction. Members of the JAK protein family contain highly conserved structural domains designated JAK homology domains (JH).

There are currently seven known JH domains (JH1-JH7). JH1 is the functional catalytic kinase domain based on sequence homology with other known tyrosine kinases. This domain consists of approximately 200 amino acid residues. For JAK2, this domain possesses a critical activation loop that becomes phosphorylated in order to activate the kinase molecule. In 1997, Feng et al. demonstrated that phosphorylation of tyrosine residue (Y1007) in the activation loop of JAK2 was essential for JAK2 activation and downstream signaling events using in vivo and in vitro kinase reactions. The model of JAK activation suggests, that in the absence of phosphorylation in the activation loop, substrates (e.g., receptor subunits) and ATP are unable to bind to the JAK molecule. However, after phosphorylation has occurred on Y1007 a conformational change in the activation loop allows substrates access to specific binding sites in the catalytic groove (Yasukawa, 1999). JH2 is a non-functional catalytic kinase domain consisting of

approximately 200 amino acid residues. Although the JH2 domain bears sequence homology to typical tyrosine kinase domains, this domain lacks catalytic activity and therefore is termed the pseudokinase domain. The functional role of this domain remains unclear; however, studies have suggested that the JH2 domain may potentially have a kinase inhibitory function. In these studies, mutations in this region leads to increased JAK2 signaling. Both the JH1 and JH2 domains are located near the carboxy terminus and comprise the major portion of the JAK molecule (Aringer, 1999; Leonard, 2001; Kisseleva et al., 2002). The JH3-JH5 domains on the JAK molecule are poorly understood and require additional work to elucidate their putative functions. JH6-JH7, amino terminal domains, have been implicated to be important in the association between the JAK molecule and the specific cytokine receptor (Aringer, 1999; Leonard, 2001; Kisseleva et al., 2002).

$JAK1^{-/-}$ mice exhibit a lethal phenotype shortly after birth probably resulting from incomplete neuronal development. $JAK1^{-/-}$ mice do not respond to cytokines important in neuronal development such as leukemia-inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). $JAK1^{-/-}$ mutant cells do not respond to IL-6, IL-11, IFNγ, IFNα, IFNβ, IL-13, IL-10, IL-2, IL-4, IL-7, IL-9, or IL-15 (Aringer et al., 1999; Leonard, 2001). $JAK2^{-/-}$ mice exhibit an embryonic lethal phenotype probably due to a block in erythropoiesis (Parganas et al., 1998; Ortmann et al., 2000; Leonard, 2001). $JAK2^{-/-}$ mutant cells do not respond to stimulatory signals from IL-3, IL-5, IFNγ, or erythropoietin. In addition to the pathology associated with $JAK3^{-/-}$ mice described earlier, $JAK3^{-/-}$ mice also show defects in lymphoid development, B cell maturation, and thymocyte proliferation. $JAK3^{-/-}$ cells fail to respond to cytokines that share the common

receptor chain, γc. These cytokines include IL-2, IL-4, IL-7, IL-9, and IL-15. Recent data regarding $TYK2^{-/-}$ mice reveal that loss of TYK2 in vivo leads to a reduction in antiviral activity. $TYK2^{-/-}$ mice are unable to clear vaccinia virus and show a reduced T cell response following a challenge with lymphocytic choriomeningitis virus (LCMV) (Karaghiosoff et al., 2000; Leonard, 2001). $TYK2^{-/-}$ cells fail to respond to IL-12 and respond weakly to IFNα, IFNβ, and IL-10.

While JAK knockout mice exhibit lethal phenotypes, cells from JAK knockout mice are viable in culture but are unresponsive to particular cytokines. The evolutionary importance of JAK molecules in development is clearly seen in studies in which the Drosophila JAK gene, hopscotch, was mutated and analyzed. Severe deformities were evident in fruit flies possessing the mutated hopscotch due to significant segmentation defects (Hou et al., 2002).

Amino acid sequence homology of the autophosphorylation site for JAK2 conducted using the basic local alignment search tool (BLAST) reveals 100% homology for the autophosphorylation site of the JAK2 kinase in other mammalian species (Table 3). The conservation observed in this region clearly indicates the importance of the autophosphorylation site in JAK2 function and provides the rationale for the suppressive functions observed in both human and mouse cells lines as demonstrated in this study.

Table 3. Sequence Comparison of the Mammalian JAK2 Autophosphorylation Site[a]

Species	Sequence
Mus musculus (house mouse)	^{1001}L P Q D K E Y Y K [V] K E P^{1013}
Homo sapien (human)	^{1001}L P Q D K E Y Y K [V] K E P^{1013}
Rattus norvegicus (Norway rat)	^{1001}L P Q D K E Y Y K [V] K E P^{1013}
Sus scrofa (pig)	^{1001}L P Q D K E Y Y K [V] K E P^{1013}

[a]Amino acid sequences of the JAK2 autophosphorylation site were retrieved from http://www.ncbi.nlm.nih.gov. Specific tyrosine residues required for JAK2 activation are shown in the rectangle (Feng et al., 1997).

Table 4. Sequence Comparision of the JAK Family Autophosphorylation Site[a]

JAK1	IETDKEYYTVKDD
JAK2	LPQDKEYYKVKEP
JAK3	LPLDKDYYVVREP
TYK2	VPEGHEYYRVRED

[a]Amino acid (13 residues) sequences for the autophosphorylation sites of the Janus kinase protein family members are aligned. Identical residues are highlighted. The underlined amino acid residues denote conserved residues. Sequence homology percentages for JAK2 and other JAK proteins are: JAK2-JAK1: 53%; JAK2-JAK3: 69%; JAK2-TYK2: 46%. Sequence alignments were analyzed using Clustal W version 1.82.

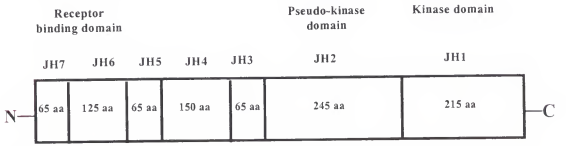


Figure 2. General Domain Schematic of the Janus Kinase (JAK) Protein Family.
The JAK tyrosine kinase protein family contains four members: JAK1, JAK2, JAK3, and TYK2. Each JAK molecule contains seven distinct regions: JH1-JH7. The JH1 domain is the functional kinase domain that following receptor binding to an appropriate ligand becomes phosphorylated on a specific tyrosine residue (Y1007). The JH2 domain is the pseudo-kinase domain. This domain is believed to play a role in the autoregulatory activities of JAK2. The JH6-JH7 domains mediate binding of JAK molecules to cytokine receptor proteins. Adapted from Shuai and Lui, 2003.

Table 4 shows a sequence comparison of the JAK autophosphorylation site for each of the JAK protein family members. JAK2 and JAK3 demonstrate the highest sequence homology.

Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription (STAT) proteins are a family of latent cytoplasmic transcription factors that act downstream of JAK activation and mediate intracellular signaling from a wide variety of cytokines, growth factors, and hormones. Currently, seven STAT proteins have been discovered in mammals: STAT1,

STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Structurally, all seven STAT proteins share six conserved domains: amino-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain, and the transactivation domain (Horvath, 2000; Kissevelar et al., 2002). STAT proteins are variable in length and contain approximately 700-900 amino acid residues.

The transcriptional activator domain (TAD) is located in the carboxy terminus and contains the appropriate tyrosine (Tyr701 – STAT1α) or serine (Ser727 – STAT1α) residue that undergoes phosphorylation by protein kinases to activate the STAT protein. The SH2 domain (Src homology 2) contains approximately 100 amino acid residues. This domain preferentially binds to phosphorylated tyrosines on proteins. The SH2 domain allows the STAT molecule to function as a docking protein where it binds to the cytoplasmic region of the cytokine receptor at a specific phosphotyrosine-containing residue (Tyr401) and activated via JAK-mediated phosphorylation on tyrosine residue Tyr701. The SH2 domain also mediates dimerization with another activated STAT protein. STATs form homodimers or heterodimers which enter the nucleus and bind DNA at specific sequences and stimulate transcription of ligand specific genes. STAT dimers preferentially bind to gamma interferon activated site (GAS) sequences, promoter element for IFNγ response genes, in the nucleus. These sites are denoted by a consensus sequence, TTNCNNNAA. The linker domain is located between the SH2 domain and the DNA-binding domain and may play a role in the regulation of transcription mediated by IFNγ. As its name would imply, the DNA-binding domain is responsible for nucleotide base recognition on the DNA molecule at specific sequences in the promoter of cytokine related genes. The coiled-coil domain of the STAT molecule is important for binding to

transcriptional regulators (e.g., IRF-9) and may be responsible for nuclear export of STAT. The amino-terminal domain plays a role in the binding affinity of STAT homodimers or heterodimers bound to the DNA molecule, nuclear translocation, and binding of transcriptional coactivators such as CBP/p300. This poorly studied domain consists of approximately 130 amino acids. Studies in which STAT genes have been mutated or targeted have provided essential details into the precise functions of these transcription factors. STAT1 knockout mice fail to respond to type I or type II interferons confirming that STAT1 proteins play a significant role in interferon mediated biological responses. STAT2 knockout mice also fail to produce a biological response to type I interferons (e.g., antiviral, gene transcription). The STAT3 knockout mouse exhibits an embryonic lethal phenotype and fails to respond to IL-6 and IL-10 signaling. STAT4 knockout mice fail to respond to IL-12 mediated signaling. STAT5a and STAT5b knockout mice fail to respond to growth hormone and prolactin stimulation respectively, while STAT6 knockout mice are defective in IL-4 and IL-13 signaling. $STAT6^{-/-}$ mice demonstrate a decrease in their ability to form Th2 T-cell populations and the ability of B-cells to class switch to the IgE isotype.

Similar to JAK kinases, STAT proteins are also evolutionarily conserved molecules and appear as homologues for mammalian STAT in several lower eukaryotic model systems including *Drosophila*, *Dictyostelium*, and *Caenorhabditis* (e.g., marelle, *Drosophila*) and have a profound effect on developmental mechanisms in these organisms.

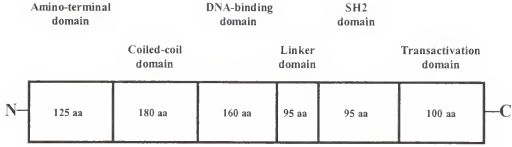


Figure 3. General Domain Schematic of the Signal Transducer and Activator of Transcription (STAT) Protein Family.
The STAT protein family contains seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. STAT molecules share similar structural homology and consist of six domains. The SH2 domain is critical for binding to the phosphorylated tyrosine residues on the cytokine receptor. The transactivation domain contains tyrosine and serine molecules that become phosphorylated following ligand stimulation leading to activation of the cytoplasmic transcription factor. The DNA-binding domain recognizes unique cytokine specific DNA sequences in the nucleus. Adapted from Shuai and Lui, 2003.

Negative Regulation of the JAK-STAT Pathway

The activity of JAK tyrosine kinases, and consequently signaling via the JAK-STAT pathway, is negatively controlled by members of the suppressors of cytokine signaling family (SOCS), also called the cytokine-inducible SH2 containing (CIS) family (12), protein inhibitors of activated stat (PIAS), phosphatases, and polyubiquination.

The SHP protein family are constitutively expressed tyrosine phosphatases containing two members: SHP-1 and SHP-2. SHP-1 has previously been shown to interact with signaling components of the B cell receptor and down regulate signaling pathways mediated by the B cell receptor (BCR) (Somani et al., 2001). Using coimmunoprecipitation studies, Wu et al. (2000) have shown that SHP-1 is associated with JAK2 in the erythropoietin-dependent leukemic cell line, UT-7/Epo, in an erythropoietin independent manner. SHP-1 has also been shown to be involved in the regulation of apoptoic signals in neutrophils and TCR signaling (Yousefi and Simon,

2003; Zhang et al., 2000). SHP-1 also inhibits cytokine activity mediated by the IL-4 receptor, IFNα, c-kit receptor, and the erythropoietin receptor. SHP-1 was first thought to play a critical role in signaling of hematopoietic cells when SHP-1 deficient mice (motheaten, *me*) were observed to demonstrate increased proliferation of mature B cells and phagocytic cells and increased antibody levels in the serum (Zhang et al., 2000). The term motheaten is derived from the patchy appearance of mice homozygous for the SHP-1 deletion mutation. SHP-2 is expressed mainly in embryonic tissue and is found to associate with a variety of signaling molecules (Tartaglia et al., 2004). SHP-2 has been shown to act both as a positive regulator (Ras/MAPK pathway) and a negative regulator (JAK-STAT pathway). STAT5 dephosphorylation has been shown to occur by interaction with SHP-2 in the nucleus. SHP-2 mutations have also been proposed to be the etiological factor leukemogenesis in humans (Tartaglia et al., 2004).

Activated STAT proteins are negatively regulated by a family of constitutively expressed proteins identified as protein inhibitors of activated STAT (PIAS). There are four known members of the PIAS family: PIAS1, PIAS3, PIASX, and PIASY. PIAS1 and PIASY have been shown to inhibit STAT1. PIAS3 has been shown to inhibit STAT3 and PIASX have previously been shown to inhibit STAT4 transcriptional activation. The mechanism for inhibition of STAT mediated transcriptional activation for PIAS1 and PIAS3 are due to direct binding to activated STAT1 and STAT3 respectively, resulting in the subsequent blockage of DNA binding (Liu et al., 1998). Lui and coworkers demonstrated decreased DNA-binding activity of activated STAT1 and a significant reduction in STAT1-mediated transcriptional activity in the presence of PIAS1. PIASX and PIASY also inhibit STAT proteins but do not act by blocking STAT-DNA binding

interactions. Experiments demonstrating the association of PIASX and histone deacetylases (HDACs), enzymes implicated in altering chromatin structure and thereby resulting in the repression of gene transcription, may represent a mechanism PIAS proteins employ to target STAT mediated transcription. Another possible mechanism for PIAS mediated inhibition of STAT proteins may be linked to small ubiquitin-like protein modifier (SUMO) ligase activity intrinsic to all PIAS proteins. SUMO modification of signaling proteins, such as STAT transcription factors appear to account for inhibition of STAT activation in a poorly understood mechanism (Rogers et al., 2003).

Another mechanism by which the JAK-STAT pathway is negatively regulated is through the process of ubiquitylation (Shuai and Liu, 2003). Ubiquitin is a highly evolutionarily conserved protein that marks proteins for degradation in the cytosol. Substrate polyubiquitination targets proteins for proteasome-mediated degradation in an ATP dependent process. Protein targeting to the proteasome is understood to primarily involve three enzymes E1, E2, and E3 (Passmore and Barford, 2004). These three enzymes act in a sequential manner to tag and direct cellular and nuclear proteins to the multiprotein proteasome complex. Specifically, E1, an ubiquitin-activating enzyme, activates ubiquitin. Activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme (Ubc), E2. Following transfer to E2, ubiquitin is believed to be transferred to a third ubiquitin-proteasome pathway enzyme E3. E3 or ubiquitin ligase participates in the transfer of ubiquitin to a specific protein (Passmore and Barford, 2004). After several rounds of ubiquitylation to a particular substrate the protein is then degraded to peptide fragments. Following peptide formation deubiquitylating enzymes are then used to recycle ubiquitin for future rounds of protein degradation events (Passmore and Barford,

2004). Earlier it was mentioned that SOCS proteins possess a SOCS box motif that has been implicated in the targeting of proteins of the JAK-STAT pathway to the proteasome for proteolysis via ubiquitination using elongin B and elongin C, proteins that associated with E3 ligase. Ungureanu et al. (2002) demonstrated that the ubiquitin-proteasome pathway regulates activated JAK2 upon cytokine signaling using immunopreciptation and protease inhibitor analysis.

Suppressors of Cytokine Signaling (SOCS) Protein Family

Currently, there are eight identified members of the inducible SOCS family, SOCS-1 to SOCS-7 and CIS. SOCS1, SOCS3, and CIS have been extensively studied while data regarding the other SOCS proteins is relatively scarce. SOCS proteins inhibit the JAK-STAT pathway by blocking activation of JAK molecules and by directly binding to JAK molecules or molecules of the cytokine receptor complex. SOCS-1 and SOCS-3 are of interest in the studies proposed here as they are the negative regulators of both JAK2 and EGFR (Alexander, 2002). SOCS-1 contains approximately 200 amino acids and has a predicted molecular weight of 24 kDa. A unique feature of SOCS proteins is that they can only bind to phosphotyrosine residues. Of the eight identified members of the SOCS protein family, relatively little is known about SOCS4-SOCS7. Although more information is known about CIS1 and SOCS2, this brief review of the SOCS protein family will primarily cover SOCS1 and SOCS3 which are the most studied SOCS proteins and considered to be more applicable to the current study. CIS or cytokine-inducible Src homology 2 (SH2) protein inhibits cytokine activity by binding to the activated receptor and competes with STAT for binding sites to the activated cytokine

receptor. By blocking STAT docking sites, activation of STAT molecules is inhibited. The resulting cytokine-mediated signal is terminated.

SOCS proteins contain three important structural domains (Figure 4). The SOCS family of regulatory proteins contains a 12 amino acid region called the kinase inhibitory region (KIR) which is located near the amino terminal domain, the centrally located SH2 domain that acts in part with KIR, and a SOCS box located near the carboxy-terminal and thought to play a role in targeting proteins bound to SOCSs for proteasomal degradation (Larsen and Ropke, 2002). An additional 12 amino acid region located downstream of the SH2 domain (extended SH2 domain) is also critical for SOCS function in cells. Mutations in isoleucine (I) at position 68 and leucine (L) at position 75 abrogated or reduced the binding between SOCS and JAK protein (Larsen and Ropke, 2002) suggesting that these two amino acid residues in the extended SH2 domain are responsible for binding affinity between the SOCS and JAK molecules. Moreover, mutations of amino acid residues at positions 56 (F), 59(F), 64(D), or 65(Y) abolish the inhibitory function and reduce binding suggesting the that these amino acids located in the KIR domain are essential for the suppressor actions of SOCS proteins (Larsen and Ropke, 2002).

It has been suggested that the KIR region structurally mimics the activation loop of the JH1 region of JAK and thereby inhibit JAK function by serving as nonfunctional analogous substrate (Larsen and Ropke, 2002). The amino terminal region contains a variable length domain, which shows very little sequence homology to other SOCS proteins. It was shown by analysis of amino acid sequence that SOCS1 and SOCS3 are more closely related to each other than other SOCS proteins.

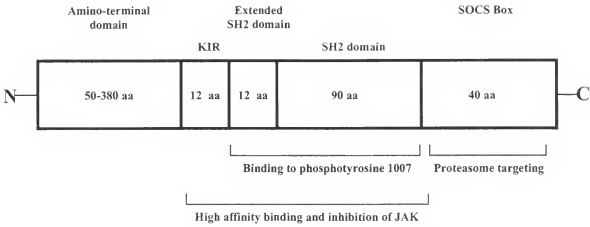


Figure 4. General Domain Schematic of the Suppressor of Cytokine Signaling (SOCS) Protein Family.
The eight identified SOCS proteins contain four distinct regions: amino-terminal domain, SH2 domain, extended SH2 domain, and the SOCS box. The central SH2 domain binds to a phosphorylated tyrosine residue on Janus kinase (JAK) or receptor proteins. A 12 amino acid region, extended SH2 domain, which extends from the SH2 domain contains essential residues for SOCS binding. SOCS proteins also contain a variable length amino-terminal region. An additional 12 amino acid region called the kinase inhibitory region (KIR) is located in the amino-terminal region and is critical for kinase inhibition. The 40-amino acid residue SOCS box is located in the carboxy terminal region and is thought to mediate proteasomal degradation of the SOCS molecule and its associated JAK kinase binding partner. Adapted from Larsen and Ropke, 2002.

Homology of primary amino acid sequence may account for overlapping biological properties (Hilton et al., 1998). Specifically, both SOCS1 and SOCS3 bind to JAK tyrosine kinases as determined using immunoprecipitation studies following cytokine stimulation of various cell lines. In addition, human SOCS proteins share high amino acid sequence similarity with both mice and rats suggesting a conserved function in other mammalian species (Larsen and Ropke, 2002).

SOCS1 and SOCS3 are primarily found in a wide variety of mammalian tissues such as spleen, lung, and liver and are relatively undetected in cells unstimulated by cytokines. However, following stimulation from a diverse array of cytokines SOCS

mRNA expression is readily detectable following analysis (Sakamoto et al., 1998; Fujimoto and Naka, 2003). Expression of SOCS1 and SOCS3 is usually rapid (30 min) and typically persists for several hours (≤ 8 hours). The mechanism for SOCS degradation is still unclear.

Emanuelli et al. (2000) showed that SOCS genes contain STAT-binding regions using electrophoretic mobility shift assays (EMSAs) providing compelling evidence that SOCS proteins are primarily induced by STAT molecules via interaction with specific SOCS genes. SOCS proteins regulate biological activity of cytokine signal transduction in negative feedback loops by inhibiting or attenuating activation of JAK or STAT molecules, reducing STAT translocation to the nucleus, reducing STAT homo- or heterodimerization, and reducing transcription of cytokine specific response genes (reviewed in Larsen & Ropke, 2002). In addition, despite their name SOCS proteins have also been shown to be induced by hormones such as insulin. Previous work has demonstrated that insulin stimulation of murine adipocytes is capable of increasing SOCS-3 mRNA expression (Emanuelli et al. 2000). Previous studies on SOCS1 and SOCS3 have demonstrated that JAK inactivation and subsequent STAT inactivation occurs by association between SOCS1 and SOCS3 and JAK. Employing transfection of truncated SOCS1 deletion mutants it was shown that SOCS1 specifically binds to the phosphorylated tyrosine residue 1007 in the activation loop of the cytoplasmic kinase protein (Figure 2). Specifically, it was shown for SOCS1 and SOCS3 that the SH2 domain, an extended SH2 domain, and the kinase inhibitory region are necessary for the inhibition of JAK-mediated signal transduction (Larsen and Ropke, 2002). These studies demonstrated that the SH2 domain is necessary for binding but alone does not contribute

to the kinase inhibitory properties of the molecule. It is now known that while SOCS1 interacts directly with JAK proteins, SOCS3 must first bind to the activated receptor subunits to mediate its suppressive effects on JAK molecules.

SOCS induction is not specific to the JAK-STAT pathway. Ohya et al (1997) demonstrated the induction and association of SOCS1 with a novel nonreceptor tyrosine kinase molecule known as Tec. Tec is believed to play a primary role in T cell function and development (Lucas et al, 2003).

Since SOCS proteins negatively regulate signal transduction pathways associated with hematopoietic cell development, differentiation, and proliferation, it is not surprising that defective SOCS proteins may play a role in a variety of diseases associated with uncontrolled cytokine signaling (Alexander, 2002). It was shown by Alexander et al. (1999) that mice lacking a functional SOCS1 gene ($SOCS1^{-/-}$) die within a month of birth with a clinical condition characterized by lymphopenia, T cell activation, and liver necrosis. However, when knockout mice lacking both SOCS1 and IFNγ are treated with antibodies specific for IFNγ they do not succumb to the same fate suggesting that the condition is due to uncontrolled IFNγ stimulation. Conversely, SOCS3 knockout mice die during embryogenesis due to defects in placental development (Roberts et al, 2001). Additionally, given the primary function of SOCS-1 to negatively regulate signaling pathways mediated by cytokine binding it is not surprising that SOCS-1 and other SOCS proteins may play key therapeutic roles in a variety of diseases associated with uncontrolled cytokine signaling (Kubo et al., 2003). An example of the use of SOCS-1 as a potential therapeutic was demonstrated in the TEL-JAK2 model system (Peeters et al., 1997; Lacronique et al., 1997; Ho et al., 1999; Monni et al., 2001; Frantsve et al., 2001;

Ho et al., 2002). TEL-JAK2 an oncogene, associated with human leukemia, arises from a chromosomal translocation leading to the fusion of the carboxyl terminal segment of the JAK2 gene and the amino terminal segment of the TEL gene. The resulting fusion product known as the TEL-JAK2 fusion protein contains the necessary domain required for JAK2 autophosphorylation (JH1) and thereby renders JAK2 constitutively active in cells leading to malignant cell proliferation. In the hematopoietic cell line Ba/F3, SOCS-1 was shown to act as a potent tumor suppressor and shown to effectively inhibit the kinase activity of the TEL-JAK2 fusion protein in vitro (Lacronique et al., 1997; Monni et al., 2001). It would be of great interest to investigate the effect of Tkip on hematopoietic cells constitutively expressing the TEL-JAK2 tyrosine kinase fusion protein.

JAK-STAT Pathway and Cancer

Previous research has provided insight into the effects of the inactivation of key molecules in the JAK/STAT pathway on cytokine signaling events (Leonard and O'Shea, 1998; Imada and Leonard, 2000). Specifically, inactivation or inhibition of specific JAK and STAT proteins produce cells and/or animals that are completely unresponsive to cytokines that rely on these proteins for cellular activity (Leonard and O'Shea, 1998; Imada and Leonard, 2000; Darnell et al., 1994). Furthermore, hyperactivated JAK/STAT proteins have been implicated in cancer or inflammatory diseases (Darnell et al., 1994; Darnell, 2002).

Knockout studies involving members of the JAK and STAT protein families offers key insights into the function of these molecules and also elucidates interesting information regarding the relationship between JAK and STAT molecules in human disease. Unlike the other JAK family members JAK3 deficiency have been implicated in

the extremely fatal severe combined immunodeficiency (SCID) in human patients. SCIDs are diseases primarily observed in children. The most prevalent form of SCIDs is associated with the X chromosome and results in severe T cell deficiency and B cell abnormalities. Since T cells and B cells are fundamental to proper functioning of the immune system, individuals with SCIDs are susceptible to simple infection that would otherwise be eliminated by a healthy immune system (Aringer et al., 1999). As already noted, JAK3 is responsible for cellular signaling mediated by a wide variety of cytokines important to T and B cell development and proliferation therefore the absence of JAK3 in hematopoietic cells readily explains the occurrence of SCIDs in patients lacking JAK3.

Mutated JAK proteins have also been known to lead to an abnormal increase in the number of leukocytes in body tissues. Several forms of leukemias such as acute lymphoblastic leukemia, chronic myelogenous leukemia, and adult T-cell leukemia have been reported to arise in part due to unregulated JAK proteins (Verma et al., 2003). Previous research (Kumar et al., 1994) has also demonstrated the involvement of JAK proteins in the activation of MAP kinases in B cells suggesting that JAK plays a pivotal role in important kinases in cell division. The significance of JAK proteins on cellular transformation was also shown by Sakia and coworkers (1997) who demonstrated that activation of JAK2 was sufficient to sustain B cell survival and mediate the induction of bcl-2, an integral membrane protein that negatively regulates proteins involved in apoptosis. Moreover, JAK participation in the development of cancer usually involves chromosomal translocations of JAK kinase genes. These abnormal translocations produce constitutively activated JAK kinases that have very dramatic and malignant consequences

in the human body. The best observed example of this phenomenon is the TEL-JAK2 fusion protein described in another section.

Constitutively activated STAT proteins have also been implicated in a variety of human carcinomas including leukemia, breast cancer, and prostate cancer. Of the seven identified STAT molecules STAT3 and STAT5 have recently been implicated to be major players in the development of oncogenesis in a variety of hematopoietic and cancer cell lines (Spiekermann et al., 2002; Dhir et al., 2002; Verma et al., 2003; Cotarla et al., 2004). STAT3 has been shown by many investigators to promote cell proliferation and cell survival by regulating apoptosis or inducing cellular transformation (Darnell et al., 1999). Earlier it was shown in experiments using electrophoretic mobility shift assays (Yu et al., 1995) that there was increased STAT3 DNA-binding activity and constitutive tyrosine phosphorylation of STAT3 in fibroblasts transformed with v-src oncogene suggesting an association between STAT3 activation and v-src induced cellular transformation. Moreover, it has been shown by several investigators that elevated STAT3 expression is correlated with prostate cancer cell and tumor survival and reduction in apoptotic signals. Antisense Stat3 nucleotides were used to inhibit the growth of human prostate cancer cells lines and reduce the level of STAT3 phosphorylation in these cell lines (Mora et al., 2002). Recently, elevated protein levels of nuclear localized phosphorylated STAT5a was observed in primary breast adenocarcinomas (Cotarla et al., 2004) using immunohistochemistry and immunoblotting analysis. These experiments demonstrate the importance of constitutively activated STAT3 and STAT5a in cancer cell growth. These experiments also offer insights into potential molecular targets for anticancer therapeutics.

Prostate Cancer

Prostate cancer is the second highest cause of cancer-related deaths in the United States. The American Cancer Society projects that nearly 230,000 men will be diagnosed with prostate cancer in 2004 and approximately 30,000 men will die from the disease in the United States. African American men are particular susceptible to the disease as incidence of prostate cancer in African American men are higher than for other ethnic groups. The cause for the disparity among different ethnic groups is still unknown. Men aged 65 or older constitute approximately 80% of all diagnosed prostate cancers. Although the precise etiological factors have not been elucidated for prostate cancer, several mechanisms have been implicated. Including environmental factors, age, genetic factors, or determined by family history. Prostate cancer symptoms may include the enlargement of the prostate, problems with urination, and lower back pain. Treatment for the disease includes hormonal treatment, chemotherapy, radiation, and surgery.

The prostate is a male reproductive glandular organ located in front of the rectum surrounding the urethra. The prostate secretes an alkaline substance that is a primary component of seminal fluid. Prostate cancer arises when cells in the prostate undergo transformations that lead to uncontrolled cell proliferation and eventual spread of cancerous cells. PSA blood screening, biopsies, and digital rectal exams (DRE) are common early diagnostic tests physicians use to diagnose prostate cancer in men which lead to an increase in survival rates for prostate cancer in the last 20 years. Prostate specific antigen (PSA), a serine protease secreted by prostate epithelial cells, is the major biological marker for prostate cancer and is commonly used in diagnosis (screening) of

the disease. PSA concentration levels of 2.5 ng/ml or higher in blood serum usually indicates the presence of prostatic cancer cells.

The major forms of prostate cancer fall into two distinguishable groups: androgen sensitive and androgen insensitive. Androgen sensitive prostate cancer arises when cancerous tissue growth is dependent on the male sex hormone androgen. Androgen insensitive prostate cancer is more serious form of the disease in which cell proliferation continues in the absence of androgen. Androgen deprivation therapy is a common treatment method in men with androgen sensitive prostate cancer. However, recent evidence shows that this therapy simply reduces the effects of the disease and that nearly all cases of androgen sensitive prostate cancer will progress to the more malignant form of prostate cancer (i.e., androgen insensitive).

Current immunological therapies are focused on treating hormone refractory prostate cancer with prostate cancer cell directed approaches without undesirable side effects associated with traditional cancer treatment methods. PC-3, LNCaP, and DU145 are the most studied human prostate cancer cell lines in prostate cancer research. PC-3 cells were originally extirpated from metastatic bone adenocarcinoma from a Caucasian male. PC-3 cells are completely (100%) tumorigenic in nude mice when inoculated subcutaneously at 10^7 cells/mouse (Kaighn et al., 1979). LNCaP prostate cancer cells were isolated from metastatic left supraclavicular lymph node carcinoma from a Caucasian male. Like PC-3 cells LNCaP cells are tumorigenic in nude mice (Horoszewicz et al., 1983). DU145 cells were isolated from metastatic brain carcinoma from a Caucasian male. DU145 cells are also tumorigenic in nude mice (Stone et al., 1978).

Targeted Approach to Cancer Therapy

A new prevailing paradigm for cancer chemotherapy is aimed at specifically targeting cellular processes, signal transduction pathways, and mutated proteins that give rise to uncontrolled cell proliferation. The goal of this therapeutic approach is to identify critical molecular components that lead to tumorigenesis and to design specific inhibitors to control their effects. There are a variety of targeted approaches to study the regulation of specific tyrosine kinases that are associated with cancer and inflammatory disorders like arthritis. These involve receptor-specific antibodies, decoy receptors to bind and inactivate ligands, small molecules with specificity towards the ATP-binding sites, and small molecules that block kinase function by unknown mechanisms. Recently, several novel anti-cancer drugs have been developed to treat cancer employing this targeted approach most notably Gleevec for the treatment of chronic myelogenous leukemia and Herceptin for the treatment of breast cancer.

Chronic myelogenous leukemia is a form of cancer that affects white blood cells of the myeloid lineage. The increase in cell proliferation of myelogenous cells in the bone marrow, blood, and tissues give rise to the clinical effects associated with the disease. Chronic myelogenous leukemia is primarily caused by generation of a constitutively active tyrosine kinase known as Bcr-Abl. The genetic basis for the Bcr-Abl tyrosine kinase stems from the formation of the Philadelphia chromosome (present in 95% of CML patients). This mutated chromosome results from a translocation between human chromosomes 22 (Bcr) and 9 (Abl). The BCR-ABL gene gives rise to the fusion of the Bcr protein and the Abl tyrosine kinase, resulting in the constitutive activation of the kinase activity of Abl. The Bcr-Abl fusion tyrosine kinase thus promotes the unregulated

cellular proliferation of certain white blood cells. Gleevec (imatinib mesylate, STI-571) is the best example of a small drug identified for the treatment of cancer, where it binds to the ATP-binding site of the oncogenic Bcr-Abl and inhibits the tyrosine kinase activity of the constitutively active tyrosine kinase known as Bcr-Abl (Shawver et al., 2001; May, 2003). This has resulted in dramatic control of chronic myelogenous leukemia (CML), and a rare form of stomach cancer where this oncogene is the determining factor for oncogenesis. Gleevec has also been demonstrated to inhibit lung cancer cell growth in vitro. Zhang and coworkers (2003) demonstrated that Gleevec effectively inhibited cell proliferation in the human lung cancer cell line A549 in a dose dependent manner. The optimal concentration (IC50) of Gleevec was shown to be 2-3 μM. The inhibitory effects observed of gleevec in this cell line are possibly due to the cross reactive properties of gleevec. In addition to binding to the Bcr-Abl tyrosine kinase fusion protein, gleevec also has been shown to bind to both c-kit and PDGF receptors.

Breast cancer is a major problem affecting women in the United States. Although a specific cause for this type of carcinoma has not been identified several factors have been purported to be consistent with the development of breast cancer such as genetic disposition, family history, and obesity (Key et al., 2004). Metastatic forms of breast cancer cells often overexpress the epidermal growth factor receptor 2 (HER2, erb2) on their cell surface. HER2 is a receptor tyrosine kinase that when activated by growth factor binding relays cellular signals that lead to cellular proliferation. Overexpression of HER2 causes cells to be abnormally stimulated resulting in increased cell growth and tumuriogenesis. HER2 related cancers proliferate at a faster rate than cancer cells in which HER2 is found at normal levels (Baselga and Hammond, 2002).

The classic and original demonstration of a monoclonal antibody that blocked a tyrosine kinase function is Herceptin (Trastuzumab), which is a humanized monoclonal antibody specific for the HER2 receptor (Shawver et al., 2001; May, 2003). Herceptin, which is administered intravenously to patients binds to the extracellular binding domain of HER2 and prevents downstream growth-promoting signals. This discovery has resulted in the treatment of aggressive forms of breast cancer where the cancer cells overexpress HER2. It seems clear from the anti-cancer approaches described above that an understanding of the molecular basis of specific cancers may lead to the production of more powerful chemotherapeutic drugs to treat cancer.

Significance of the Study

Tyrosine kinases are important biological molecules involved in signal transduction events and are essential in normal cellular functions. Defective tyrosine kinases are common etiological factors for cancer and inflammatory diseases. The past decade has seen an increase in research aimed at identifying tyrosine kinase markers for detection of cancer and for developing tyrosine kinase inhibitors that counteract the biological effects observed in autoimmune diseases and malignant cancers.

The objectives of the current study are to characterize Tkip by determining the binding specificity of Tkip, examining the effects of Tkip on IFNγ mediated signal transduction mechanisms, and investigating the effects of Tkip on IFNγ mediated biological functions. The aim of this study is to focus on the unique specificity of Tkip for the autophosphorylation site of JAK2, which is directly involved in IFNγ signaling. The major hypothesis of this work is that Tkip will inhibit JAK2 activation by binding to its autophosphorylation site and thus inhibit or alter the ability of JAK2 to transduce

appropriate cellular signals induced by IFNγ. Tkip may serve as a useful therapeutic for cancer and inflammatory disorders.

CHAPTER 2
MATERIALS AND METHODS

Peptides

The peptides used in the study are listed in Table 5, and were synthesized in the laboratory of Dr. Howard M. Johnson on a Perseptive Biosystems 9050 automated peptide synthesizer using fluorenylmethyloxycarbonyl (Fmoc) chemistry as described previously (Chang and Meienhofer, 1978). Biotinylation and lipophilic modifications (Lipo) to peptides were made at the N-terminus (Szente et al., 1996). The addition of a lipophilic group (palmitoyl-lysine) to the N-terminus of the synthetic peptide facilitates peptide entry into cells. Peptides were characterized by mass spectrometry and purified by high performance liquid chromatography (HPLC). Following HPLC analysis each peptide profile was shown to display a single peak. Peptides were dissolved in either deionized water, phosphate buffered saline (PBS), or dimethyl sulfoxide (Sigma, St. Louis, MO) at 20 mg/ml, and stored at –20 °C until use. Peptides were dissolved in either buffer or complete growth media before use.

Cell Culture and Viruses

PC-3 human prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in F12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere in 150 cm^2 tissue culture flasks. WEHI-3 murine macrophages were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.05 M β-mercaptoethanol at 37 °C in a 5% CO_2 atmosphere in 75 cm^2 tissue culture flasks. WISH human fibroblast cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere in 150 cm^2 tissue culture flasks. Bovine aortic endothelial cells (BAECs) were generously provided by Drs. R. Johnson and T. Nakagawa of the Division of Nephrology, College of Medicine, University of Florida. BAEC culture reagents were purchased from Cambrex (Walkersville, MD). BAECs were cultured according to the manufacturer's instructions in six-well tissue culture plates at 37°C in a 5% CO_2 atmosphere. Starvation medium contains Eagle's MEM and RPMI 1640 without antibiotics. Encephalomyocarditis virus (EMCV) was obtained from ATCC and stored at –70 °C until use. For bioassays EMCV was used at a multiplicity of infection (MOI) of 0.02.

Binding Assay

Tkip, Tkip5A, IFNγ(95-125), CDK-2 cyclin box (CDK), and vascular endothelial growth factor receptor-1 (VEGFR-1) peptides were incubated in 96-well (Nunc, Polysorp, Rochester, NY) plates overnight at 4 °C in 0.1 M carbonate binding buffer at a final concentration of 3 μg/ml. Following overnight incubation, peptides were dried to the wells of the microtiter plate. Wells were then washed three times with wash buffer containing 0.9% NaCl and 0.05% tween-20 in Dulbecco's phosphate buffered saline (PBS) and blocked with 2% gelatin and 0.05% tween-20 in PBS for 2 h at room temperature to block nonspecific binding. Wells were then washed three times with wash buffer and incubated with various concentrations (10 mM, 5 mM, 2 mM, 0.7mM, 0.2mM,

0.07 mM, 0.02 mM, 0.007 mM) of biotinylated JAK2 WT peptide and biotinylated p-JAK2 WT peptide for 1 h at room temperature in blocking buffer. Following incubation wells were washed five times with wash buffer. Biotinylated peptides were detected by incubation with 1:500 dilution of Neutravidin™ biotin binding-protein conjugated with horseradish peroxidase (Molecular Probes, Eugene, OR) in blocking buffer for 1 h at room temperature. Wells were then washed five times with wash buffer to remove unbound conjugates. A solution of o-phenylenediamine (OPD – HRP substrate) in stable peroxidase buffer (Pierce, Rockford, IL) was used to develop the reaction. 2 M H_2SO_4 was used to halt color development. Absorbance was detected using a 450 microplate reader (Bio-rad, Hercules, CA) at 490 nm. Control experiments were carried out as described above in the absence of immobilized peptides.

Competition Experiments

For competition experiments 3 μg Tkip was plated and incubated in 96-well plates (Nunc, Polysorp, Rochester, NY) overnight at 4 °C. Following washing steps (0.9% NaCl and 0.05% tween-20 in PBS) and blocking in blocking buffer (2% gelatin and 0.05% tween-20 in PBS) for 2 hr at room temperature, unlabeled CDK, VEGFR-1, JAK2WT peptides were incubated for 30 min at room temperature. A final concentration of 0.7 mM biotinylated JAK2 WT peptide and biotinylated p-JAK2 WT peptide were incubated for 1 hr in each well to determine extent of competition with unlabeled peptides. Unlabeled peptides were added at 10-fold excess (7mM) of the biotinylated JAK2 WT peptides. Binding was detected by incubation with Neutravidin™ biotin binding-protein conjugated with horseradish peroxidase (1:500, Molecular Probes,

Eugene, OR) in blocking buffer for 1 h at room temperature followed by incubation with OPD peroxidase solution. The reaction was stopped using 2 M H_2SO_4.

In vitro Kinase Assays

Autophosphorylation activity of EGFR and VEGFR-1 was measured in a reaction mixture containing kinase buffer (20 mM Tris-HCl [pH 7.5], 2 mM dithioreitol, 50 mM potassium chloride, 0.3 mM sodium orthovanadate, 5 mM magnesium chloride, 10 mM glycerophosphate, 2 mM EGTA, 1 M manganese chloride), Tkip, substrates, and 5 μCi of [^{32}P]γ-ATP (specific activity 6000 Ci/mmol; 1 mCi = 37 Mbq) (Amersham Biosciences, Piscataway, NJ). EGFR and VEGFR in vitro kinase assays were carried out in 22.5 μL reaction volumes containing 10 μL Tkip, 2.5 μL substrates, and 10 μL [^{32}P]γ-ATP incubated at 30°C for 10 min. EGFR (supplied precomplexed with EGF) was obtained from Upstate Biotechnology (Lake Placid, NY). VEGFR and VEGF were obtained from Calbiochem (San Diego, CA) and Peprotech (Rocky Hill, NJ), respectively.

Determination of JAK2 autophosphorylation activity was performed in reaction mixtures containing recombinant human JAK2 immobilized on agarose beads (Upstate Biotechnology, Lake Placid, NY), 1 μCi/μl of [^{32}P]γ-ATP (Amersham Biosciences, Piscataway, NJ), and either Tkip or JAK 2 WT peptide incubated in kinase buffer (10 mM HEPES [pH 7.4], 50 mM sodium chloride, 0.1 mM sodium orthovanadate, 5 mM magnesium chloride, 5 mM manganese chloride). JAK2 kinase assays were performed in 35 μL reaction volumes containing 10.5 μL Tkip, 20 μL JAK2 agarose beads, 1 μL sIFNGR-1, and 3.5 μL [^{32}P]γ-ATP incubated at 30 °C for 30 min with intermittent agitation. It was determined in our laboratory that addition of a mouse soluble IFNGR-1 subunit dramatically stimulated JAK2 kinase activity and, hence, was added at 2 μg per

reaction. EGFR, VEGFR, and JAK2 kinase reactions were terminated with the addition of 5 µl 6X SDS sample buffer (0.5 M Tris-HCl [pH 6.8], 36% glycerol, 10% SDS, 9.3% dithioreitol, 0.012% bromophenol blue).

Incubation of JAK2 agarose beads in SDS sample buffer (100 °C) was designed to elute bound proteins from the agarose beads. The reaction mixtures were separated on a 10% SDS polyacrilamide gel. Autoradiography was used to determine phosphorylation activity.

Src kinase activity was performed with a Src substrate peptide (KVEKIGEGTYGVVYK) using a Src kinase assay kit according to the manufacturer's specifications (Upstate Biotechnology, Lake Placid, NY). Briefly, Src substrate peptide was incubated in Src kinase buffer (100 mM Tris-HCl [pH 7.2], 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.02 mM Na_3VO_4, and 2 mM dithioreitol), 5 µCi of [^{32}P]γ-ATP (75 mM $MnCl_2$, 500 µM ATP), recombinant human Src (Upstate Biotechnology, Lake Placid, NY), and Tkip, JAK2 WT peptide, or in the absence of peptide for 10 min at 30 °C. Reaction mixtures were spotted on P81 phosphocellulose discs (supplied with kit) to bind phosphorylated Src substrate peptide, washed three times for 5 min with 0.75% phosphoric acid, washed twice with acetone for 1 min, and placed in vials to which 4 ml of ScintiVerse® (Fisher, Pittsburgh, PA) was added. The phosphocellulose discs were analyzed using a liquid scintillation counter to measure ^{32}P-labeled proteins. For comparison with Src, JAK2 was also assayed using the same procedure. The assay was setup as described above for the Src kinase assay. However, reactions were incubated in the presence of 2 mM dithioreitol in the buffer to release JAK2 from the agarose beads. Following the appropriate incubation period reactions were gently centrifuged.

Supernatants were spotted on phosphocellulose discs and processed as above. Unless otherwise stated the peptides used in the kinase reactions described above were used at 50 μM. Immunoblotting was performed in parallel to determine protein levels used in the kinase reactions. Dose response kinase activity studies of JAK2 and EGFR were performed as described above.

Antiproliferation Assay

PC-3 cells were plated (2 x 10^5 cells/ml) in 6-well plates and incubated with various concentrations of Tkip and MuIFNγ(95-125) (50 μM, 25 μM, 12 μM, 6 μM, 3 μM, 1 μM, 0.5 μM, 0 μM) for 5 days at 37 °C in complete media in a 5% CO_2 atmosphere. Following incubation, cells were trypsinized, centrifuged, and resuspended in 100 μL of complete media. Cells were then enumerated directly using a hemocytometer under a light microscope. Cell viability was assessed using trypan blue (0.4%, Invitrogen, Carlsbad, CA) exclusion. Trypan blue dye exclusion is a test of plasma membrane physical integrity. The biological stain is excluded from crossing intact plasma membranes and therefore stains dead cells. Cell viability was expressed as the percentage of live cells versus the total (live/dead) number of cells. Direct counts of untreated cells were used to assess percent inhibition. Values are expressed as the mean ± standard deviation of independent experiments performed in duplicate.

Immunoblot Analysis

WISH fibroblast cells from the third passage were plated in 6-well plates at a cell density of 3 x 10^6 cells/well. After overnight incubation with complete culture media WISH cells were incubated in starvation medium for 17 hours and pretreated with complete culture media or different concentrations of Tkip (8 μM or 1 μM) for an additional 17 hours at

37 °C in a 5% CO_2 atmosphere in complete media. WISH cells were then incubated in the presence or absence of 5000 U/ml IFNγ (PBL Biomedical Laboratories, Piscataway, NJ) to activate the JAK-STAT pathway. Fibroblast cells were washed twice in cold phosphate buffered saline (PBS) to remove residual media and cell debris. Cells lysates were prepared by adding 200 µl of cold lysis buffer (50 mM Tris-HCl, 0.25 M NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM PMSF, 10% glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 0.25% sodium deoxycholate, 1% NP-40, and 0.1% SDS) to each well. Lysis was allowed to proceed for 1hr at 4 °C (rocking) to ensure complete lysis. Lysates were then centrifuged at 10, 000 rpm for 10 min to remove cell debris and the supernatant was transferred to a fresh microcentrifuge tube. Samples containing lysate, lysis buffer, and sample buffer were heated (100 °C) boiled for 5 min and pulsed centrifuged. Protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel (Biorad, Richmond, CA). Proteins were then transferred to nitrocellulose membranes (Amersham Biosciences) overnight at low voltage. To reduce nonspecific binding membranes were incubated in blocking buffer containing 5% nonfat dry milk in PBS for 1 hr at ambient temperature and washed in wash buffer containing 1% nonfat dry milk and 0.1% Tween-20 in PBS (PBST) three times. To detect phosphorylated STAT1, membranes were incubated with antibodies to tyrosine-phosphorylated STAT1 (Cell Signaling Technologies, Beverly, MA) phosphorylated at tyrosine residue 701 (1:100) in wash buffer overnight with agitation at 4 °C. Polyclonal p-STAT1 antibodies were generated by immunizing rabbits with a synthetic peptide (KLH coupled) specific for human STAT1 (p-Tyr 701). To detect

phosphorylated JAK2, membranes were incubated with antibodies to tyrosine-phosphorylated JAK2 (Upstate Biotechnology) phosphorylated at tyrosine residues 1007/1008 (1:100) in wash buffer overnight with agitation at 4 °C. Antibodies for p-JAK2 were generated by immunizing rabbits with a synthetic peptide (KLH coupled) specific to amino acid residues 1002-1013 of murine JAK2 (p-Tyr 1007/1008). After three washes in PBST, membranes were incubated in HRP-conjugated goat anti-rabbit IgG secondary antibodies (Santa Cruz, Santa Cruz, CA) at a dilution of 1:5000 in wash buffer for 1 hr at ambient temperature. Following three washes in wash buffer, membranes were incubated for 1 min with enhanced chemiluminescence detection reagents (Amersham), exposed to photographic film to visualize protein bands.

Flow Cytometry

WISH fibroblast cells (2×10^6) were incubated for 48 h with media alone, 25 μM MuIFNγ(95-125) peptide, or lipophilic Tkip (LT) at varying concentrations (1 μM, 10 μM, 25 μM) in the presence or absence of 5000 units/ml IFNγ in 6-well culture plates at 37 °C in a 5% CO_2 atmosphere. Following incubation, cells were washed twice with PBS and harvested by trypsinization into two sets of 5 ml round bottom polystyrene tubes (Fisher, Pittsburgh, PA) and washed twice with PBS. For cell surface staining, a direct immunofluorescence protocol was employed. Briefly, cells were incubated on ice with a 100 μl staining solution in PBS of either anti-human MHC class I monoclonal antibody conjugated to R-phycoerythrin (R-PE, 1:100) or with monoclonal mouse IgG2a antibody conjugated to R-PE (1:100) as an isotype control for 1 h at room temperature in the absence of light. The fluorescent-conjugated isotype antibody was used to determine background fluorescence by identifying nonspecific binding of the monoclonal antibody

to WISH cells. The above antibodies were purchased from Ancell Corporation (Bayport, MN). Following incubation, cells were then washed three times with PBS to remove unbound antibody molecules. WISH fibroblast cells were finally resuspended in 500 µl of PBS and analyzed on a FACScan fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). The apparatus was equipped with an argon laser. The red fluorescence of R-PE was excited at 488 nm. Cell debris was isolated from intact cells by performing forward and side scatter analysis on each sample and therefore excluded from the analysis. The fluorescence intensity of the negative controls were subtracted from the mean fluorescence for the R-PE labeled cells. For each sample 10,000 stained cells were examined. Flow cytometry data were analyzed using CellQuest analysis software (Becton Dickinson, San Jose, CA).

Antiviral Assay

Antiviral activity was determined using a standard viral cytopathogenic effect assay described previously with minor modifications (Szente et al., 1996). Antiviral assays were performed to evaluate the ability of Tkip to block antiviral activity mediated by IFNγ. Briefly, WEHI-3 murine macrophage cells ($5x10^5$) were incubated with either media alone, 2000 U/ml IFNγ, or both 2000 U/ml IFNγ and 10 µM lipophilic Tkip for 24 h in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 37 °C in a 5% CO_2 atmosphere. Following incubation, WEHI-3 cells were washed three times with growth media and infected with EMCV for 1 h at 37 °C. EMCV was added at a multiplicity of infection (MOI) of 0.02. WEHI-3 cells were then washed three times to remove viral particles and incubated in fresh growth media for an additional 24 h at 37 °C. Plates were subsequently blotted dry and stained with 0.1% crystal violet solution for

5 min to stain live cells. Unbound crystal violet was aspirated and the plates were thoroughly rinsed with deionized water, blotted, and allowed to air dry. Plates were then scanned using an Astra 2100U flatbed computer scanner (UMAX Technologies, Dallas, TX) and analyzed using ImageJ 1.29 software (NIH) to assess cell survival. Percentages of cell survival were determined by comparing experimental treatment groups with the virus only control group. Recombinant murine IFNγ (specific activity 1 x 10^7 U/ml) used in the antiviral assay described above were obtained from PBL (Camarillo, CA).

Immunoprecipitation

Bovine aortic endothelial cells were plated at a density of $3x10^6$/well in 6-well plates and allowed to incubate for 10 hrs at 37 °C. Growth media was then removed and replaced with growth media with or without peptides at the indicated concentrations overnight at 37 °C. Cells were then treated with serum-free medium alone or serum-free medium containing peptides at the indicated concentrations for 2 hrs. BAECs were then incubated in the presence or absence of 50 ng/ml VEGF (Upstate Biotechnology) in serum-free media for 10 min and lysed with 500 µl lysis buffer. Cells were lysed in lysis buffer containing 50 mM Tris-HCl [pH 7.4], 0.25 M NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM PMSF, 10% glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 0.25% sodium deoxycholate, 1% NP-40, and 0.1% SDS for 1 hr at 4 °C while rotating. Lysates were microcentrifuged at 7,000 x g at 4 °C for 15 min to remove cell deris and nuclei. Supernatants were transferred to a new microtube and incubated with 1 µg/ml anti-VEGFR-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hrs at 4 °C while rotating. Protein G-Sepharose beads (40 µL, 1:1 slurry) were added to the

supernatant and allowed to incubate for 1 hr at 4 °C while rotating. Following centrifugation to pellet the protein G immune complexes, supernatant was removed and discarded. The immune complexes were subsequently washed three times with lysis buffer and twice with PBS. For SDS-PAGE analysis, immune complexes were boiled (100 °C) in 35 μl of SDS sample buffer for 5 min and resolved on a 12% polyacrylamide gel. Following transfer to nitrocellulose membranes, membranes were blocked, washed, and treated with specific antibodies to detect phosphorylated tyrosine proteins (4G10, Upstate Biotechnology) and VEGFR-2 proteins. Detection of proteins was accomplished using enhanced chemiluminescence protein detection reagents (Amersham).

Cell Cycle Analysis

DU145 and LNCaP cells (2 x 10^6) were plated in 6-well plates overnight in complete growth medium to allow cells to adhere to the plate. Cells were then incubated in starvation medium for 48 h at 37 °C in a 5% CO_2 atmosphere to allow cellular synchronization in the G_0/G_1 phase. Cells were then treated with media alone, Tkip or DMSO in media for 24 h at 37 °C. Following treatments, cells were washed twice with PBS, harvested by trypsinization for 5 min, neutralized with complete growth media, and collected in 5-ml round bottom polystyrene tubes (Fisher, Pittsburgh, PA). Cells were then washed three times with PBS and resuspended in 100 μl of sample buffer (1% glucose in PBS) to which 1 ml of ice-cold ethanol was added and incubated for 30 min on ice. Following the fixation step, cells were then washed three times with sample buffer to remove ethanol and finally resuspended in sample buffer containing 45 μg/ml propidium iodide solution and 45 U/ml RNase A (Sigma, St. Louis, MO). Cells were incubated for 1 h at room temperature in the absence of light and analyzed by fluorescent-activated cell

sorting on a FACSort (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed using ModFit LT™ software (Topsham, ME).

CHAPTER 3
RESULTS

Tyrosine Kinase Inhibitor Peptide (Tkip)

The autophosphorylation site of JAK2 consists of residues ^{1001}LPQDKEYYKVKEP with ^{1007}Y as the tyrosine autophosphorylation residue that results in activation of JAK2 (Feng et al., 1997). The focus was on the complementary peptide approach to develop a short peptide capable of binding to this site (Villian et al., 2000). This approach was used to develop peptides that bind to the neuropeptide arginine vasopressin (Johnson and Torres, 1988). The complementarity refers to the hydropathic complementarity, which has been shown empirically to result in peptide/peptide binding (Villian et al., 2000). Recently, an algorithm has been developed that specifies the "best" complementarity fit (Fassina et al., 1992). The sequences of several peptides varied in their complementarity to ^{1001}LPQDKEYYKVKEP were generated. It was discovered that the best complementary fit did not necessarily result in the best binding to JAK2 peptide (data not shown). Thus, the best binding which was not the best complementary fit occurred with complementary peptide WLVFFVIFYFFR. This peptide was developed by reading the complementary strand codons in the JAK2 autophosphorylation site in the 5'-3' direction as originally described in detail by others (Blalock and Bost, 1986). The sequences of the peptides used are presented in Table 5.

Tkip Binds to the Autophosphorylation Site of JAK2

Data on binding of WLVFFVIFYFFR to [1001]LPQDKEYYKVKEP, as determined by ELISA, are presented in Figure 5. Biotinylated JAK2 autophosphorylation peptide

Table 5. Amino Acid Sequences of the Peptides Used in this Study.

Peptide[a]	Sequence
Tkip	WLVFFVIFYFFR
JAK2 WT	[1001]LPQDKE**Y**YKVKEP
p-JAK2 WT	[1001]LPQDKE**Y**YKVKEP
JAK1	[1016]IETDKE**Y**YTVKDD
TYK2	[1048]VPEGHE**Y**YRVRED
VEGFR-1	[1208]SSDVR**Y**VNAFKFM
CDK-2 cyclin box	[41]KTEGVPSTAIREISLLKELNH
MuIFNγ(95-125)	[95]AKFEVNNPQVQRQAFNELIRVVHQLLPESSL

[a]Peptides were synthesized as described in Materials and Methods. Murine IFNγ sequence is derived from the mature form. Lipophilic group modifications were added to the N-terminus of the peptide. Tyrosines targeted for phosphorylation are indicated in bold. Tkip = tyrosine kinase inhibitor peptide (12 amino acids), JAK2 = Janus Kinase 2, JAK1 = Janus Kinase 1, TYK2 = Tyrosine Kinase 2, WT = wild type, P = phosphorylated, VEGFR-1 = vascular endothelial growth factor receptor-1, CDK = cyclin dependent kinase. The square denotes the phosphotyrosine moiety. The JAK2 WT sequence is the same for both mice and humans.

(biotinylated JAK2 WT) was added at different concentrations to solid-phase complementary peptide, designated tyrosine kinase inhibitory peptide Tkip, as well as to solid-phase control peptides. These control peptides consisted of IFNγ sequence 95-125 (IFNγ 95-125), cyclin-dependent kinase, cyclin box peptide 41-61 (CDK 41-61), and vascular endothelial growth factor receptor autophosphorylation peptide VEGFR 1208-1222. As shown in Figure 5A, JAK2 WT bound only to Tkip in a dose-dependent manner. Binding to the control peptides was neglible. In ELISA competitions, JAK2 WT but neither CDK 41-61 nor VEGFR 1208-1222, inhibited biotinylated JAK2 WT binding

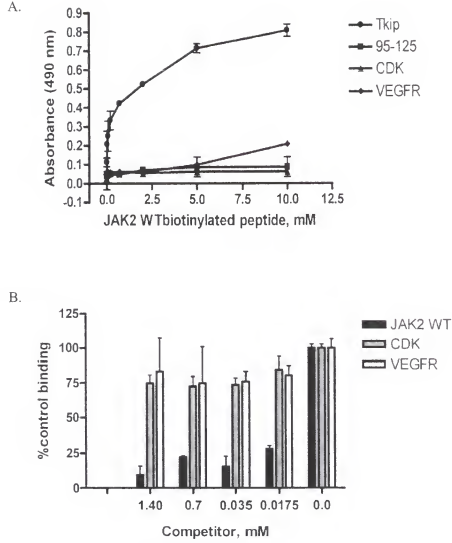


Figure 5. Binding of Tkip by JAK2 Autophosphorylation Peptide, JAK2 WT.
(A) Direct binding of wild type JAK2 WT peptide (see Table 5 for sequences) to Tkip. The wild type JAK2 peptide was synthesized with a biotin group incorporated at its N-terminus during peptide synthesis, and the peptide purified. Biotinylated JAK2 WT peptide, at the indicated concentrations, was added in triplicate to wells of 96-well plates coated with either Tkip, VEGFR peptide, CDK-2 cyclin box peptide, or MuIFNγ(95-125) peptide (see Table 5 for sequences). Wells were blocked with PBS + 2% gelatin + 0.1% Tween-20. The assay was developed using standard ELISA methods using a neutravidin-HRP conjugate to detect bound biotinylated wild type JAK2. Non-specific binding was determined from wells that were not coated with any peptide to which the same concentrations of biotinylated peptide were added.
(B) Biotinylated wild type JAK2 peptide was bound to Tkip coated on 96-well plates, either in the absence (100% binding) or presence of indicated concentrations of JAK2 WT peptide, VEGFR peptide or an unrelated peptide [MuIFNγ(95-125)]. Bound biotinylated wild type JAK2 peptide was detected by ELISA using a neutravidin-HRP conjugate. The data are representative of at least two separate experiments.

to Tkip (Figure 5B). The binding data suggest that the Tkip peptide specifically recognized JAK2 WT.

Tkip Inhibits Kinase Activity of JAK2

Since Tkip was originally synthesized to abolish JAK2 activity, it was necessary to determine whether Tkip could inhibit JAK2 autophosphorylation as well as phosphorylation of IFNγ receptor subunit IFNGR-1 in vitro. In the autophosphorylation assay, Tkip was incubated with radiolabeled ATP, full length JAK2 protein and IFNGR-1. Following incubation, samples were subjected to SDS-PAGE and autoradiography to assess kinase activity. As shown in Figure 6, Tkip at 50 μM inhibited both the autophosphorylation of JAK2 as well as JAK2 phosphorylation of IFNGR-1. A control peptide, JAK2 WT, at the same concentration had no effect on JAK2-induced tyrosine phosphorylations.

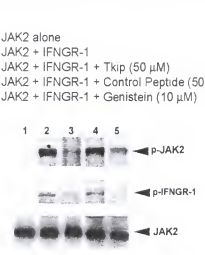

1. JAK2 alone
2. JAK2 + IFNGR-1
3. JAK2 + IFNGR-1 + Tkip (50 μM)
4 JAK2 + IFNGR-1 + Control Peptide (50 μM)
5 JAK2 + IFNGR-1 + Genistein (10 μM)
1 2 3 4 5
p-JAK2
p-IFNGR-1
JAK2

Figure 6. Kinase Inhibitory Specificity of Tkip.
Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring JAK2 autophosphorylation. Kinase reactions were subjected to SDS-PAGE and the gels dried. Dried gels were subjected to autoradiography to detect ^{32}P-labeled proteins (*upper and middle panels).* The negative control peptide was the JAK2 WT peptide used at the same concentration. Genistein, a nonspecific inhibitor of JAK2, was used as a positive control. Total JAK2 protein was measured from separate reactions that were subjected to SDS-PAGE and the proteins Western transferred to a nitrocellulose membrane followed by detection with standard immunoblotting and ECL detection protocols (*bottom panel*). The data are representative of at least two separate experiments.

Thus, consistent with Tkip binding to JAK2 WT, it also inhibited JAK2 autophosphorylation as well as JAK2 phosphorylation of IFNGR-1. It should be noted that JAK2 autophosphorylation itself was dependent on the presence of the IFNGR-1 receptor subunit in the reaction mixture. Thus, Tkip inhibited the kinase activity of JAK2.

Tkip Does Not Inhibit Kinase Activity of VEGFR or c-Src

Tkip was next tested for its inhibitory effects against several other tyrosine kinases. VEGFR is involved in the development and growth of the vascular endothelial system (Ferrara et al., 2003). As shown in Figure 7, Tkip at 50 μM did not inhibit the autophosphorylation of VEGFR, but under the same conditions completely inhibited JAK2 autophosphorylation as well as JAK2 phosphorylation of IFNGR-1 (Figure 10). Thus, compared to VEGFR, Tkip shows specificity toward JAK2. This is consistent with the failure of Tkip to bind to VEGFR autophosphorylation site as per Figure 5.

Next Tkip was tested against a nonautophosphorylation tyrosine kinase, c-src. As shown in Figure 7, Tkip at 50 μM failed to inhibit c-src phosphorylation of a protein substrate. By contrast, Tkip significantly blocked JAK2 autophosphorylation/IFNGR-1 phosphorylation as estimated by greater than 95% inhibition of ^{32}P incorporation into JAK2/IFNGR-1 (Figure 8B). Thus, the data on Tkip failure to block VEGFR and c-src tyrosine phosphorylations are evidence of specificity of Tkip for inhibition of JAK2 autophosphorylation via interaction with the JAK2 autophosphorylation site.

Tkip Inhibits Kinase Activity of EGFR

Interaction of Tkip with the JAK2 autophosphorylation site and inhibition of JAK2 function raises the question of possible functional relationship of Tkip to a group

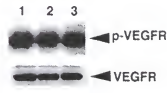


Figure 7. Tkip Does Not Inhibit Autophosphorylation of VEGFR.
Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring VEGFR autophosphorylation. The control peptide was the JAK2 WT peptide (see Table 5) used at the same concentration. Kinase reactions were subjected to SDS-PAGE and the gels dried. Dried gels were subjected to autoradiography to detect ^{32}P-labeled proteins (*upper panel*). Total VEGFR protein was measured from separate reactions that were subjected to SDS-PAGE and the proteins Western transferred to a nitrocellulose membrane followed by detection with standard immunoblotting and ECL detection protocols (*lower panel*).

of regulators called suppressors of cytokine signaling or SOCS. SOCS are recently discovered negative regulators of cytokines, growth factors, and hormone signaling (reviewed in Kile et al., 2002; Alexander, 2002; Larsen and Ropke, 2002; Hanada et al., 2003). Currently, there are eight identified members of the SOCS family, SOCS-1 to SOCS-7 and CIS. SOCS-1 and SOCS-3 are of interest to the current studies as they are the negative regulators of both JAK2 and the epidermal growth factor receptor (EGFR) (reviewed in Kile et al., 2002; Alexander, 2002; Larsen and Ropke, 2002; Xia et al., 2002; Hanada et al., 2003). Therefore, it was next determined whether Tkip could inhibit EGFR tyrosine kinase activity. As shown in Figure 9 Tkip at 50 μM completely inhibited EGFR autophosphorylation. For comparison, Tkip also inhibited JAK2 autophosphorylation as

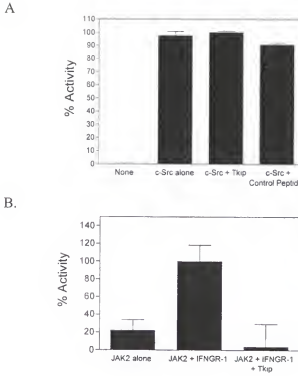


Figure 8. Tkip Does Not Inhibit Tyrosine Phosphorylation Activity of c-Src.
(A) Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring c-src tyrosine phosphorylation of a synthetic substrate peptide. C-src kinase activity was determined using a kit purchased from Upstate Biotechnology (Lake Placid, NY). The control peptide (*c-src + Control peptide*) was the JAK2 WT peptide used at the same concentration. *None* represents reactions without c-src or peptides as a measure of background. Triplicate samples of the kinase reactions were spotted on P81 cellulose discs, and processed as described by the manufacturer. The discs were counted for radioactivity, and kinase activity is reported as percentage of the activity of the reaction containing neither Tkip nor control peptide (*c-src alone*), after subtraction of background (*None*).
(B) Tkip inhibition of kinase reactions for JAK2 were setup as described in Figure 6 as a positive control, but samples were processed as in 2C. Activity is reported as percentage of activity in reactions containing JAK2 and IFNGR-1 alone, after subtraction of background.

well as JAK2 phosphorylation of IFNGR-1 (Figure 10). Thus, Tkip inhibited EGFR autophosphorylation, which is consistent with its specificity for the SOCS-1 and SOCS-3 autophosphorylation sites of JAK2 and EGFR. It should be kept in mind that EGFR autophosphorylation is complex with up to five autophosphorylation sites (Wells, 1999). Thus, just how SOCS-1 and SOCS-3 recognize EGFR is currently not known.

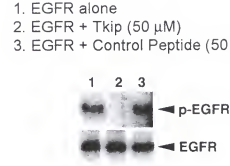


Figure 9. Tkip Inhibits Autophosphorylation of EGFR.
Tkip was added at 50 μM, where indicated, to in vitro kinase assays measuring EGFR autophosphorylation. The negative control peptide, 50 μM, was the same as in Figure 2A (JAK2 WT, see Table 5). Kinase reactions were subjected to SDS-PAGE and the gels dried. Dried gels were subjected to autoradiography to detect ^{32}P-labeled proteins (*upper panel*). Total EGFR protein was measured from separate reactions that were subjected to SDS-PAGE and the proteins Western transferred to a nitrocellulose membrane followed by detection with standard immunoblotting and ECL detection protocols (*lower panel*).

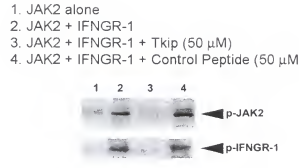


Figure 10. Positive Control for In Vitro Kinase Assay.
Samples were set up and run as in Figure 6. This experiment was run in parallel with that of Figures 7 and 9.

Tkip Inhibits Kinase Activity of JAK2 and EGFR in a Dose Dependent Manner

We next compared Tkip inhibition of JAK2 and EGFR autophosphorylation in a dose-response study. As shown in Figure 11, Tkip similarly inhibited autophosphorylation of JAK2 (Figure 11A) and EGFR (Figure 11B) with 25 to 50 μM significantly blocking JAK2 autophosphorylation and 12 to 25 μM blocked IFNGR-1 phosphorylation by JAK2, while 6 to 12 μM significantly blocked EGFR phosphorylation. Thus, the patterns of dose-response inhibition of JAK2 and EGFR were similar. It is of interest that Tkip inhibited JAK2 phosphorylation of IFNGR-1 at a lower concentration than that for JAK2 autophosphorylation itself. This suggests that Tkip can block JAK2 phosphorylation of a substrate (IFNGR-1) more effectively than the autophosphorylation of JAK2, which may reflect the possibility that Tkip binds phosphorylated JAK2 more effectively than it does unphosphorylated JAK2.

Tkip Preferentially Binds to the Activated JAK2 Autophosphorylation Site

The binding data of Figure 5 involved JAK2 WT that was not phosphorylated at ^{1007}Y. JAK2 WT recognition by SOCS-1 has been shown to involve phospho ^{1007}Y (Kile et al., 2002). Thus, Tkip does not need phosphorylation of ^{1007}Y in order to bind to JAK2 WT. We were, however, interested in determining the relative binding of Tkip to JAK2 WT unphosphorylated versus phosphorylation at ^{1007}Y (p-JAK2 WT). As shown in Figure 12, Tkip bound both JAK2 WT and p-JAK2 WT in a dose-dependent manner, but binding was most efficient to p-JAK2 WT. Fifty percent endpoint concentrations were approximately 9-fold lower for p-JAK2 WT binding versus unphosphorylated JAK2 WT binding. Thus, phosphorylation of ^{1007}Y enhances Tkip binding to the JAK2 autophosphorylation site.

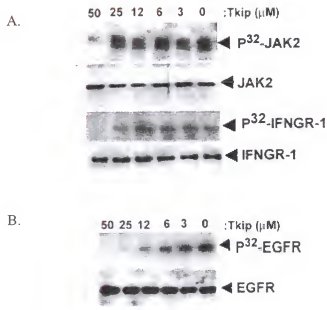


Figure 11. Dose Response of Tkip Inhibition of JAK2, IFNGR-1, and EGFR Phosphorylation In Vitro.
(A) Tkip was incubated with JAK2, IFNGR-1, and ^{32}P-ATP for 30 min at 30 °C at the indicated concentrations. The kinase reaction was resolved on a 10% SDS-PAGE. The gel was dried and exposed to photographic film for 1 hr at –70 °C to detect phosphorylated proteins (upper panel). Kinase reaction mixtures were subjected to immunoblotting with a probe specific for JAK2 and IFNGR-1 as an internal protein loading control (second and fourth panel).
(B) Tkip was incubated with EGF, EGFR, and ^{32}P-ATP for 10 min at 30 °C at the indicated concentrations. The kinase reaction was resolved on a 10% SDS-PAGE. The gel was dried and exposed to photographic film for 1 hr at –70 °C to detect phosphorylated proteins (upper panel). Kinase reaction mixtures were subjected to immunoblotting with a probe specific for EGFR as an internal protein loading control (lower panel). The data are representative of at least two separate experiments.

Tkip Inhibits STAT1α Activation Not Activation of VEGFR

It has been firmly established that tyrosine phosphorylation of STAT1α at a specific tyrosine residue (Tyr 701) is required for the activation, dimerization, nuclear translocation, and subsequent downstream biological effects of IFN-γ stimulation (Boehm et al., 1997). To assess whether Tkip could inhibit intracellular STAT1α activation we investigated the effect of Tkip on STAT1α tyrosine phosphorylation in

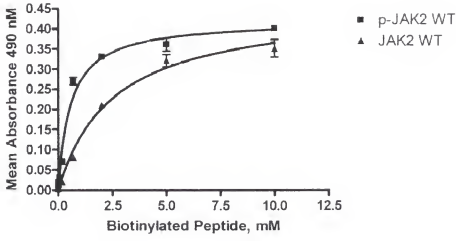
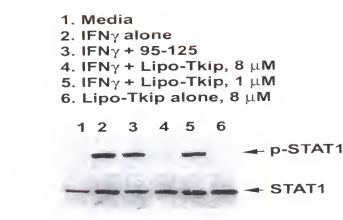


Figure 12. Binding of Unphosphorylated JAK2 WT Peptide Versus Phosphorylated JAK2 WT Peptide to Tkip.
Various concentrations of biotinylated unphosphorylated JAK2 WT (JAK2 WT) and biotinylated phosphorylated JAK2 WT (p-JAK2 WT) peptides were incubated in the presence of immobilized Tkip. Binding was measured by solid phase binding assays. The data are representative of two independent experiments performed in triplicate. Binding of phosphorylated JAK2 WT peptide versus unphosphorylated JAK2 WT peptide was found to be statistically significant ($p < 0.005$) by Student's t-test.

human fibroblast WISH cells. Cells were treated with lipophilic Tkip and IFNγ as indicated in Figure 13A and whole cell lysates were examined using immunoblot analysis with antibodies specific for STAT1α and phosphorylated STAT1α. Cells pretreated with 8 μM Tkip for 17 hr and subsequently stimulated with 5000 U/ml IFNγ for 30 min showed complete abolishment of IFNγ induced STAT1α tyrosine phosphorylation. Cells pretreated with 1 μM Tkip and stimulated with IFNγ showed no effect on STAT1α phosphorylation, suggesting that low concentrations of Tkip are not sufficient to inhibit IFNγ induced phosphorylation of STAT1α. A lipophilic irrelevant peptide, MuIFNγ(95-125), was used to show that the results observed were not dependent solely on the

lipophilic modification of Tkip. Tyrosine phosphorylation of STAT1α was not affected in cells treated with IFNγ in the absence of Tkip. As expected STAT1α phosphorylation was not observed in the absence of IFNγ treatment. STAT1α protein levels in each treatment group were monitored by reprobing the membrane with anti-STAT1α antibodies. By contrast Tkip, under the same conditions, failed to inhibit VEGFR activation in bovine aortic endothelial cells as determined by autophosphorylation of VEGFR (Figure 13B).

A.



B.

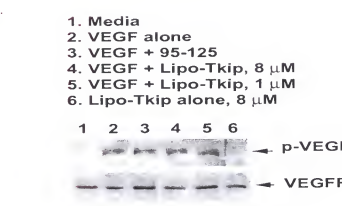


Figure 13. Tkip Inhibits IFNγ Induced STAT1α Activation in WISH Cells But Does Not Inhibit VEGF Induced Activation of VEGFR in Bovine Aortic Endothelial Cells (BAECs).

(A) Serum-starved WISH cells were pretreated with media alone or Tkip (8 μM or 1 μM) for 17 hr. Following 30 min incubation in the presence or absence of 5000 U/ml IFNγ, cells were washed, harvested, and lysed. Whole cell extracts were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and examined using specific antibodies to phosphorylated STAT1α (p-Tyr 701, upper panel). The membrane was striped and reprobed with antibodies specific to unphosphorylated STAT1α to demonstrate equal protein loading (lower panel). Data are representative of at least two separate experiments. (B) BAECs were pretreated with media alone or Tkip (8 μM or 1 μM) overnight prior to 2 hr incubation in serum free media. Following starvation, BAECs were treated with or without 50 ng/ml of VEGF for 15 min in the presence or absence of various concentrations of Tkip (8 μM or 1 μM). VEGFR phosphorylation was monitored using immunoprecipation (anti-VEGFR) and immunoblot (anti-pY) analysis. The membrane was striped and reprobed with antibodies specific to VEGFR to demonstrate equal protein loading (lower panel). Data are representative of at least two separate experiments.

These results clearly demonstrate the ability of Tkip to inhibit IFNγ mediated intracellular phosphorylation of STAT1α at the level of the cell.

Tkip Inhibits Antiviral Activity

Functionally, since Tkip inhibits JAK2 autophosphorylation and subsequent phosphorylation of IFNGR-1, and phosphorylation of STAT1α, one would predict that Tkip would inhibit IFNγ induced antiviral activity. Accordingly, we infected WEHI-3 cells with encephalomyocarditis (EMC) virus and protected the cells against EMC virus cytopathogenic effects (CPE) with 2000 U/ml mouse IFNγ. Treatment of WEHI-3 cells with 10 μM Tkip (lipophilic for cell membrane penetration) along with IFNγ resulted in approximately 75% reduction in IFNγ antiviral activity as per increased CPE of EMC virus (Figure 14). A lipophilic control peptide failed to affect IFNγ antiviral activity, so the lipophilic Tkip effect was due to Tkip. Thus, consistent with inhibition of JAK2 tyrosine kinase activity, Tkip blocked the antiviral effects of IFNγ.

Tkip Inhibits MHC Class I Upregulation

Another well-established function of IFNγ is the upregulation of MHC class I molecules on cells. Accordingly, we treated human WISH cells with 5000 U/ml of human IFNγ, which resulted in over 3-fold increase in MHC class I as per FACS analysis (Figure 15). Lipophilic Tkip at 10 μM or 25 μM completely blocked upregulation of MHC class I expression, and in fact reduced the baseline constitutive expression of MHC class I molecules. A control lipophilic peptide (MuIFNγ(95-125) had no effect. Thus, in addition to inhibition of IFNγ antiviral activity, Tkip also inhibited IFNγ upregulation of MHC class I molecules.

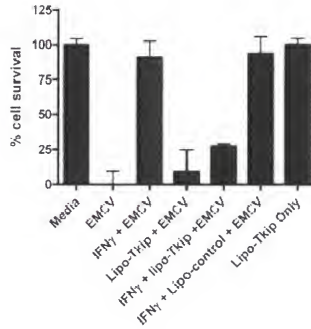


Figure 14. Tkip Inhibits the Antiviral Activity of IFNγ Against EMC Virus on WEHI-3 Cells.

WEHI-3 cells were preincubated either in the absence of peptide (*Media*), in the presence of IFNγ (2000 U/ml) alone, or IFNγ + 10 μM of lipopeptides as indicated, for 24 hr. The control lipopeptide was the lipophilic version of JAK2 WT (see Table 5 for sequence). Cells were then challenged with EMC (encephalomyocarditis) virus for another 24 hr. Cells were then stained with crystal violet, the dye extracted and the absorbance measured. Values are normalized percentages of cell survival determined by setting cells treated with EMC virus alone (*EMCV*) as 0% and cells with not virus treatment (*Media*) as 100%. The data are representative of at least two separate experiments. The difference in cell survival for cells incubated in the presence (IFNγ + EMCV + Lipo-Tkip) or absence (IFNγ + EMCV) of Lipo-Tkip was found to be statistically significant ($p < 0.005$) by Student's t-test.

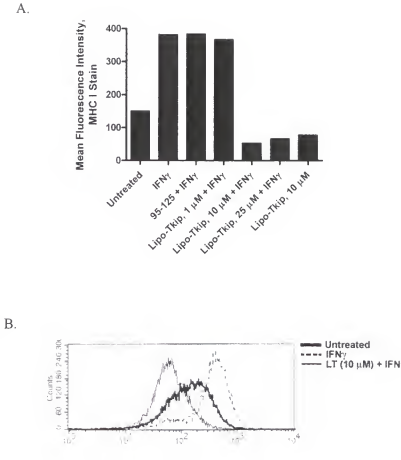


Figure 15. Downregulation of IFNγ Induced Cell Membrane Expression of MHC Class I on WISH Cells Using Tkip.

(A) WISH cells were treated in the presence or absence of IFNγ (5000 U/ml) and various concentrations of Lipo-Tkip (LT) for 48 h. Cells were then stained with R-PE conjugated monoclonal antibody specific for human MHC class I. R-PE conjugated mouse IgG2a was used as an isotype control. The data are presented as mean fluorescence intensity.

(B) Overlapping histograms indicate untreated cells (bold line), IFNγ treated cells (dashed line), and LT + IFNγ treated cells (thin line).

The fact that Tkip inhibited murine IFNγ antiviral activity on WEHI cells (Figure 14) and human IFNγ upregulation of MHC class I molecules on human WISH cells (Figure 15) is consistent with the autophosphorylation sites of murine and human JAK2 (Table 5) consisting of the same amino acid sequence.

Effects of Tkip on STAT3 Activation in Human Prostate Cancer Cell Lines

DU145 prostate cancer cells have constitutively activated STAT3 as a result of autocrine IL-6 production (Smith et al., 2001). JAK2 is involved in STAT3 phosphorylation in cells by IL-6, and SOCS-1 has been shown to inhibit this phosphorylation (Fujimoto and Naka, 2003). Treatment of DU145 cells with 8 μM lipo-Tkip blocked constitutive phosphorylation of STAT3 (Figure 16A). STAT3 is not constitutively phosphorylated in LNCaP prostate cancer cells, since this cancer cell line does not constitutively produce IL-6 (Lou et al., 2000). We treated LNCaP cells with IL-6 in a dose-response fashion and observed maximum phosphorylation of STAT3 with 10 ng/mL IL-6 (Figure 16B). Lipo-Tkip at 10 μM blocked STAT3 phosphorylation (Figure 16B). Thus, Tkip blocked constitutive (endogenous IL-6) and exogenous IL-6 induced STAT3 phosphorylation in prostate cancer cell lines. These results contain interesting implications for both cancer and inflammation, and they demonstrate that Tkip shows SOCS-1 mimetic activity in inhibition of IL-6 induced phosphorylation of the transcription factor STAT3 (Fujimoto and Naka, 2003).

Tkip Inhibits Cell Cycle Progression in Human Prostate Cancer Cell Lines

IL-6 induced STAT3 activation has been implicated in cell proliferation of prostate cancer cell lines (Lou et al., 2000; Smith et al., 2001). To test whether Tkip could inhibit cell cycle progression, prostate cancer cells (DU145 and LNCaP cell lines)

were serum-starved in serum free media for 48 h to synchronize cells in the G1 phase of the cell cycle, and then cultured for 24 h in complete growth medium to initiate cell cycling in the presence or absence of increasing concentrations of Tkip, or control peptide MuIFN-γ(95-125). Lipophilic versions of Tkip and the control peptide were used in these experiments. Following peptide treatment, cells were harvested and cell cycle progression was assessed using propidium iodide staining. T_0 represents cells harvested following serum-starvation and are an indication of the extent of synchronization of cells in G_0/G_1 before peptide treatment.

The data in Table 6 shows the effects of Tkip on DU145 prostate cancer cells. Synchronized cells stimulated with complete growth medium in the absence of peptide showed progression into the cell cycle by 24 h. In contrast, cells treated with Tkip showed a dose dependent effect of inhibition of cell cycle progression into the S phase, with almost complete inhibition at 10 μM (similar percent of cells in G_0/G_1 as the cells at T_0). Control peptide at 10 μM had no effect on cell cycle progression. As shown in Table 7, essentially similar results were obtained with LNCaP prostate cancer cells when treated with the same concentrations of Tkip. Thus, Tkip inhibits the cell cycling, and hence the proliferation, of both DU145 and LNCaP prostate cancer cells.

Tkip Inhibits Antiproliferative Activity of PC-3 Prostate Cancer Cells

To investigate the antiproliferative effects of Tkip on the human prostate cancer cell line PC-3, standard cell enumeration protocols using tyrpan blue exclusion were employed following incubation for 120 hr. For appropriate controls, cells were treated with a lipophilic version of MuIFNγ(95-125) at the same concentrations. Percent cell

viability demonstrated the dose response effect of Tkip on PC-3 prostate cancer cell proliferation (Table 8).

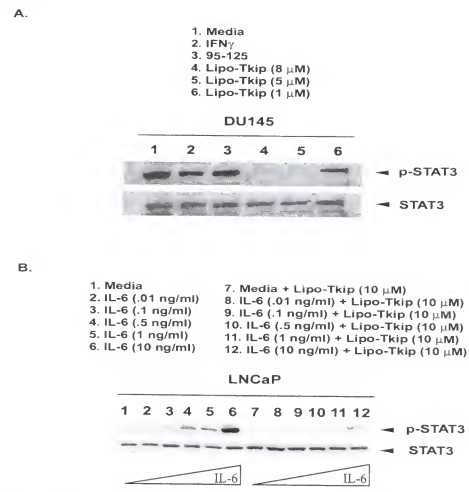


Figure 16. Effects of Tkip on Human Prostate Cancer Cell Lines DU145 and LNCaP.
(A) Tkip blocks constitutive STAT3 activation in DU145 cells. Cells were treated in the presence or absence of Tkip for 20 h at the indicated concentrations. Cell lysates were immunoblotted with polyclonal antibodies specific for phosphorylated STAT3 (Tyr 705). Determination of equal protein loading was performed by probing membranes for STAT3 after striping membrane.
(B) Tkip blocks IL-6 induced STAT3 activation in LNCaP cells. Cells were treated with varying doses of IL-6 in the presence and absence of Tkip for 20 h. Cell lysates were immunoblotted with polyclonal antibodies specific for phosphorylated STAT3 (Tyr 705) as above. Determination of equal protein loading was performed by probing membranes for STAT3 after striping membrane. Data are representative of two independent experiments.

Table 6. Effects of Tkip on the Cell Cyc le of DU145 Prostate Cancer Cells.

	Cell Cycle Phase (%) [a]		
	G_0/G_1	S	G_2/M
Media (T_0)	90.0	4.9	5.1
Media	68.6	20.6	10.8
MuIFNγ(95-125) (10 μM)	69.3	26.7	4.0
Tkip (1 μM)	69.8	24.9	5.3
Tkip (5 μM)	70.5	27.1	2.4
Tkip (10 μM)	85.5	12.8	1.7

[a] Data are representative of two independent experiments. Statistical significance was shown by Students's *t*-test between percent G_0/G_1 phase for cells treated with control lipophilic peptide and Tkip at 10 μM ($p < 0.05$).

Table 7. Effects of Tkip on the Cell Cycle of LNCaP Prostate Cancer Cells.

	Cell Cycle Phase (%) [b]		
	G_0/G_1	S	G_2/M
Media (T_0)	82.2	7.4	10.4
Media	68.8	13.0	18.2
MuIFNγ(95-125) (10 μM)	65.0	28.9	6.1
Tkip (1 μM)	62.7	34.3	3.0
Tkip (5 μM)	72.7	24.3	3.0
Tkip (10 μM)	83.0	13.7	3.3

[b] Data are representative of two independent experiments. Statistical significance was shown by Students's *t*-test between percent G_0/G_1 phase for cells treated with control lipophilic peptide and Tkip at 10 μM ($p < 0.05$).

Table 8. Inhibition of PC-3 Cell Proliferation by Tkip.

Peptide	Concentration (μM)	Total No. of Cells (x 50, 000)	Inhibition[a] (%)
Tkip	0	298 ± 2.6	—
	.5	289 ± 0.7	3.0
	1	288.1 ± 1.7	3.3
	3	183.5 ± 2.8	38.4
	6	175 ± 2.8	41.3
	12	107 ± 1.5	64.1
	25	22.7 ± 2.5	92.4
	50	6.7 ± 1.5	97.8
MuIFNγ(95-125)	0	255 ± 3.0	—
	.5	254.2 ± 2.0	0.3
	1	253.9 ± 3.1	0.4
	3	253.7 ± 3.5	0.5
	6	252.7 ± 2.1	0.9
	12	251.7 ± 2.5	1.3
	25	248.3 ± 2.1	2.6
	50	246 ± 2.6	3.5

[a]Asynchronized PC-3 cells (2×10^5) were incubated for 5 days with increasing concentrations of Tkip in complete media. Cells were harvested and counted using trypan blue exclusion. Results are expressed as mean number of cells ± standard deviation from several experiments. Tkip and MuIFNγ(95-125) were modified by adding a lipophilic group (palmitic acid) to the N-terminus of the peptide to enhance cellular uptake.

CHAPTER 4
DISCUSSION

The JAK kinases play a central role in regulation of the biological activity of hormones and cytokines. JAK1 and JAK2 are the key tyrosine autophosphorylation kinases for mediation of signal transduction of IFNγ (Kotenko and Petstka, 2000). This study presents data on the development of a 12-residue peptide, Tkip that binds to the autophosphorylation site of JAK2. Tkip blocks both JAK2 autophosphorylation as well as phosphorylation of IFNγ receptor subunit IFNGR-1. Consistent with binding to the autophosphorylation site of JAK2 and inhibition of autophosphorylation as well as IFNGR-1 tyrosine phosphorylation, Tkip also blocked both the antiviral activity and upregulation of MHC class I molecules on cells treated with IFNγ. Tkip inhibition of biological activity of IFNγ was not associated with toxicity of the cells.

By binding to the tyrosine autophosphorylation site of JAK2, Tkip represents a novel approach to specific control of tyrosine kinases. Based on the data presented in this study, and previous experimental investigations in Dr. Howard M. Johnson's laboratory, the following general mechanistic model of inhibition of IFNγ-mediated signaling by Tkip is proposed in Figure 17. The proposed model suggests that inhibition of IFNγ-mediated cellular functions may be due to a block in STAT1 activation leading to defective downstream signal transduction events and a reduction of specific biological functions associated with binding of IFNγ to the IFNγ receptor complex. Future investigations are needed to clarify the precise mechanism of STAT1 inactivation.

It is possible that Tkip binds to phosphorylated JAK2 and prevents complete JAK2 activation, thereby preventing STAT1 activation. This has potential implications for regulation of inflammatory conditions and cancer where unregulated tyrosine kinases play a central role in the resultant pathology. The fact that Tkip was designed to recognize the autophosphorylation site of JAK2 suggests a potential relationship to at least some members of a family of regulatory proteins called suppressors of cytokine signaling or SOCS.

SOCS are recently discovered negative regulators of cytokines, growth factors, and hormone signaling (Kile et al., 2002; Alexander et al., 2002, Larsen and Ropke, 2002; Hanada et al., 2003). Currently, there are eight identified members of the SOCS family, SOCS-1 to SOCS-7 and CIS. SOCS-1 and SOCS-3 are of interest here as they are the negative regulators of JAK2 as well as several other cytokine and hormone receptor systems including EGFR (Kile et al., 2002; Alexander et al., 2002, Larsen and Ropke, 2002; Xia et al., 2002; Hanada et al., 2003). In this regard, Tkip inhibited EGFR autophosphorylation, but VEGFR and c-src tyrosine kinases, which are not regulated by SOCS-1 and SOCS-3, were not affected by Tkip (Iwamoto et al., 2000). Consistent with this, the VEGFR autophosphorylation site did not bind Tkip.

Initially it was shown that Tkip bound to JAK2 autophosphorylation site where ^{1007}Y of that site was not phosphorylated. This differs from SOCS-1 recognition, where such binding occurs only when ^{1007}Y is phosphorylated (Yasukawa et al., 1999).

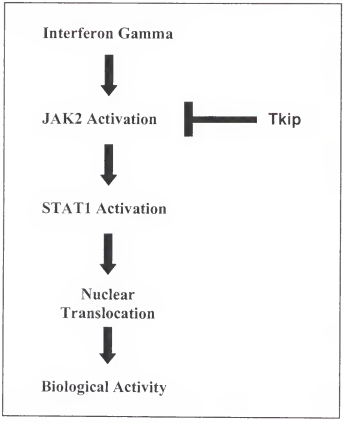


Figure 17. Proposed Model of Inhibition of IFNγ Mediated Signaling by Tkip.
This generalized model illustrates the mechanism of Tkip inhibition of IFNγ-induced biological functions. The binding of Tkip to phosphorylated JAK2 may block downstream activation of STAT1 in the cytoplasm. For simplicity, this model does not show essential intermediate signaling events associated with the JAK-STAT pathway. The precise mechanism of STAT1 inactivation by Tkip is currently not known as indicated by the question mark in the model.

However, while Tkip does bind to JAK2 autophosphorylation site in its nonphosphorylation state, we showed here that binding increased significantly when ^{1007}Y was phosphorylated, indicating that Tkip has stronger affinity for the phosphorylated ^{1007}Y at the JAK2 autophosphorylation site. Thus, there appear to be both similarities and differences in how Tkip and SOCS-1 recognize the JAK2 autophosphorylation site.

SOCS play an essential physiological role in the maintenance of homeostasis. SOCS-1$^{-/-}$ mice, for example, develop normally through embryogenesis but die within three weeks of a syndrome characterized by severe lymphopenia, fatty degeneration, necrosis of liver cells, and extensive macrophage infiltration of internal organs (Starr et al., 1998). Similarly, SOCS-3$^{-/-}$ mice initially develop normally, but soon show marked liver erythropoiesis with resultant embryonic death (Marine et al., 1999; Roberts et al., 2001). The above defects appear to occur primarily as a result of unregulated IFNγ and erythropoietin signaling. The persistent IFNγ activity in the SOCS-1 $^{-/-}$ mice is associated with abnormally activated T cells (Alexander, 2002). The unregulated presence of IFNγ results in extensive apoptosis. Evidence for the role of IFNγ in the above condition is demonstrated by injecting SOCS-1$^{-/-}$ mice with antibodies to IFNγ, which results in abolishment of premature death, and a more normal appearance of internal organs (Larsen and Ropke, 2002). It would thus be of particular interest to determine if Tkip can protect mice against the SOCS-1$^{-/-}$ lethal mutation. Both SOCS-1 and SOCS-3 are involved in regulation of JAK2, and the fact that both SOCS are needed would suggest that they act cooperatively in regulating overall JAK2 activity. SOCS-3 binds to the erythropoietin receptor with a K_d of about 1-10 μM (Hortner et al., 2002). This is similar

to the effective concentrations of Tkip for inhibiting IFNγ antiviral and MHC Class I upregulation functions shown here. These data would suggest that we have shown proof-of-concept in the first reported development of a SOCS-1 mimetic.

Given the primary function of SOCS-1 to negatively regulate signaling pathways mediated by cytokine binding, it is not surprising that SOCS-1 and other SOCS proteins may play key therapeutic roles in a variety of diseases associated with uncontrolled cytokine signaling (Kubo et al., 2003). An example of the use of SOCS-1 as a potential therapeutic was demonstrated in the TEL-JAK2 model system (Peeters et al., 1997; Lacronique et al., 1997; Ho et al., 1999; Monni et al., 2001; Frantsve et al., 2001; Ho et al., 2002). TEL-JAK2 an oncogene, associated with human leukemia, arises from a chromosomal translocation leading to the fusion of the carboxyl terminal segment of the JAK2 gene and the amino terminal segment of the TEL gene. The resulting fusion product known as the TEL-JAK2 fusion protein contains the necessary domain required for JAK2 autophosphorylation (JH1) and thereby renders JAK2 constitutively active in cells leading to malignant cell proliferation. In the hematopoietic cell line Ba/F3, SOCS-1 was shown to act as a potent tumor suppressor and shown to effectively inhibit the kinase activity of the TEL-JAK2 fusion protein in vitro (Peeters et al., 1997; Lacronique et al., 1997; Ho et al., 1999; Monni et al., 2001; Frantsve et al., 2001; Ho et al., 2002). It would thus be of great interest to investigate the effect of Tkip on hematopoietic cells constitutively expressing the TEL-JAK2 tyrosine kinase fusion protein.

Finally, the regions of SOCS-1 that are thought to bind to the autophosphorylation site of JAK2 involve a small domain, called the kinase inhibitory region (KIR), and a segment of a SH2 domain adjacent to KIR (Yasukawa et al., 1999). The sequences of

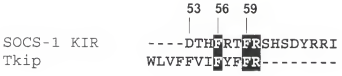


Figure 18. Alignment of the KIR of SOCS-1 and Tkip.
Identical residues are highlighted. Alignment was made to maximize identity. The numbers are for the positions of the residues in the KIR of SOCS-1, as outlined in Yasukawa et al., 1999.

Tkip and KIR are compared for homology in Figure 18. Sequence identity is seen with two F and R residues. Mutation analysis of the KIR of SOCS-1 have previously shown that F^{56} and F^{59} were critical for KIR binding to JAK2 with F^{59} being the most important residue (Yasukawa et al., 1999, Kubo et al., 2003). These residues are conserved in both Tkip and SOCS-1. Future studies using amino acid substitution should better define Tkip specificity for JAK2 as well as the relationship of Tkip regulation to that of SOCS-1.

REFERENCE LIST

Alexander, W.S. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. Nat. Rev. Immunol. *2*, 410-416.

Alexander, W.S., Starr, R., Fenner, J.E., Scott, C.L., Handman, E., Sprigg, N.S., Corbin, J.E., Cornish, A.L., Darwiche, R., Owczarek, C.M., Kay, T.W., Nicola, N.A., Hertzog, P.J., Metcalf, D., Hilton, D.J. (1999). SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell *98*, 597-608.

Aringer, M., Cheng, A., Nelson, J.W., Chen, M., Sudarshan, C., Zhou, Y.J., and O'Shea, J.J. (1999). Janus kinases and their role in growth and disease. Life Sci. *64*, 2173-2186.

Bach, E.A., Aguet, M., and Schreiber, R.D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu. Rev. Immunol. *15*, 563-591.

Barton, B.E., Karras, J.G., Murphy, T.F., Barton, A., and Huang, H.F. (2004). Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. Mol. Cancer Ther. *3*, 11-20.

Baselga, J., and Hammond, L.A. (2002). HER-targeted tyrosine-kinase inhibitors. Oncology *63 Suppl 1*, 6-16.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.

Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). Cellular responses to interferon-gamma. Annu. Rev. Immunol. *15*, 749-795.

Bogdan, S., and Klambt, C. (2001). Epidermal growth factor receptor signaling. Curr. Biol. *11*, R292-295.

Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E., Jr. (1999). Stat3 as an oncogene. Cell *98*, 295-303.

Calo, V., Migliavacca, M., Bazan, V., Macaluso, M., Buscemi, M., Gebbia, N., and Russo, A. (2003). STAT proteins: from normal control of cellular events to tumorigenesis. J. Cell Physiol. *197*, 157-168.

Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. (2002). Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat. Rev. Drug Discov. *1*, 493-502.

Chang, C.D., and Meienhofer, J. (1978). Solid-phase peptide synthesis using mild base cleavage of N alpha-fluorenylmethyloxycarbonylamino acids, exemplified by a synthesis of dihydrosomatostatin. Int. J. Pept. Protein Res. *11*, 246-249.

Cotarla, I., Ren, S., Zhang, Y., Gehan, E., Singh, B., and Furth, P.A. (2004). Stat5a is tyrosine phosphorylated and nuclear localized in a high proportion of human breast cancers. Int. J. Cancer *108*, 665-671.

Culig, Z., Bartsch, G., and Hobisch, A. (2002). Interleukin-6 regulates androgen receptor activity and prostate cancer cell growth. Mol. Cell Endocrinol. *197*, 231-238.

Darnell, J.E., Jr. (1997). STATs and gene regulation. Science *277*, 1630-1635.

Darnell, J.E., Jr. (2002). Transcription factors as targets for cancer therapy. Nat. Rev. Cancer *2*, 740-749.

Darnell, J.E., Jr., Kerr, I.M., and Stark, G.R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science *264*, 1415-1421.

Dhir, R., Ni, Z., Lou, W., DeMiguel, F., Grandis, J.R., and Gao, A.C. (2002). Stat3 activation in prostatic carcinomas. Prostate *51*, 241-246.

Duhe, R.J., and Farrar, W.L. (1998). Structural and mechanistic aspects of Janus kinases: how the two-faced god wields a double-edged sword. J. Interferon Cytokine Res. *18*, 1-15.

Endo, T.A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., Yoshimura, A. (1997). A new protein containing an SH2 domain that inhibits JAK kinases. Nature *387*, 921-924.

Feng, J., Witthuhn, B.A., Matsuda, T., Kohlhuber, F., Kerr, I.M., and Ihle, J.N. (1997). Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol. Cell Biol. *17*, 2497-2501.

Familletti, P.C., Rubinstein, S., and Pestka, S. (1981). A convenient and rapid cytopathic effect inhibition assay for interferon. Methods Enzymol. *78*, 387-394.

Fassina, G., Cassani, G., and Corti, A. (1992). Binding of human tumor necrosis factor alpha to multimeric complementary peptides. Arch. Biochem. Biophys. *296*, 137-143.

Ferrara, N., Gerber, H.P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat. Med. *9*, 669-676.

Fitzgerald, K.A. (2001). The cytokine factsbook. In Factsbook series; (Academic Press).

Frantsve, J., Schwaller, J., Sternberg, D.W., Kutok, J., and Gilliland, D.G. (2001). Socs-1 inhibits TEL-JAK2-mediated transformation of hematopoietic cells through inhibition of JAK2 kinase activity and induction of proteasome-mediated degradation. Mol. Cell Biol. *21*, 3547-3557.

Fujimoto, M., and Naka, T. (2003). Regulation of cytokine signaling by SOCS family molecules. Trends Immunol. *24*, 659-666.

Gallin, J.I., Farber, J.M., Holland, S.M., and Nutman, T.B. (1995). Interferon-gamma in the management of infectious diseases. Ann Intern Med *123*, 216-224.

Giordanetto, F., and Kroemer, R.T. (2002). Prediction of the structure of human Janus kinase 2 (JAK2) comprising JAK homology domains 1 through 7. Protein Eng. *15*, 727-737.

Giordanetto, F., and Kroemer, R.T. (2003). A three-dimensional model of Suppressor of Cytokine Signalling 1 (SOCS-1). Protein Eng. *16*, 115-124.

Hanada, T., Kinjyo, I., Inagaki-Ohara, K., and Yoshimura, A. (2003). Negative regulation of cytokine signaling by CIS/SOCS family proteins and their roles in inflammatory diseases. Rev. Physiol. Biochem. Pharmacol. *149*, 72-86.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem. J. *374*, 1-20.

Hilton, D.J., Richardson, R.T., Alexander, W.S., Viney, E.M., Willson, T.A., Sprigg, N.S., Starr, R., Nicholson, S.E., Metcalf, D., and Nicola, N.A. (1998). Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc. Natl. Acad. Sci. USA *95*, 114-119.

Ho, J.M., Beattie, B.K., Squire J.A., Frank D.A., and Barber, D.L. (1999). Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling. Blood 93, 4354-4360.

Ho, J.M., Nguyen, M.H., Dierov, J.K., Badger, K.M., Beattie, B. K., Tartaro, P., Haq, R., Zanke, B.W., Carroll, M.P., and Barber, D.L. (2002). TEL-JAK2 constitutively activates the extracellular signal-regulated kinase (ERK), stress-activated protein/Jun kinase (SAPK/JNK), and p38 signaling pathways. Blood 100:1438-1448.

Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P. (1983). LNCaP model of human prostatic carcinoma. Cancer Res. *43*, 1809-1818.

Hortner, M., Nielsch, U., Mayr, L.M., Heinrich, P.C., and Haan, S. (2002). A new high affinity binding site for suppressor of cytokine signaling-3 on the erythropoietin receptor. Eur. J. Biochem. *269*, 2516-2526.

Horvath, C.M. (2000). STAT proteins and transcriptional responses to extracellular signals. Trends Biochem. Sci. *25*, 496-502.

Hou, S.X., Zheng, Z., Chen, X., and Perrimon, N. (2002). The Jak/STAT pathway in model organisms: emerging roles in cell movement. Dev. Cell *3*, 765-778.

Imada, K., and Leonard, W.J. (2000). The Jak-STAT pathway. Mol. Immunol. *37*, 1-11.

Iwamoto, T., Senga, T., Naito, Y., Matsuda, S., Miyake, Y., Yoshimura, A., and Hamaguchi, M. (2000). The JAK-inhibitor, JAB/SOCS-1 selectively inhibits cytokine-induced, but not v-Src induced JAK-STAT activation. Oncogene *19*, 4795-4801.

Isaacs A, Lindenmann J. (1957). Virus interference. I. The Interferon. Proc. R. Soc. London Ser. *B147*, 256-267.

Ishihara, K., and Hirano, T. (2002). Molecular basis of the cell specificity of cytokine action. Biochim Biophys Acta *1592*, 281-296.

Johnson, H.M., and Torres, B.A. (1988). A novel arginine vasopressin-binding peptide that blocks arginine vasopressin modulation of immune function. J. Immunol. *141*, 2420-2423.

Johnson, H.M., Bazer, F.W., Szente, B.E., and Jarpe, M.A. (1994). How interferons fight disease. Sci. Am. *270*, 68-75.

Kaighn, M.E., Narayan, K.S., Ohnuki, Y., Lechner, J.F., and Jones, L.W. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest. Urol. *17*, 16-23.

Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., Pfeffer, K., and Muller, M. (2000) Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity 13, 549-560.

Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A., and Travis, R.C. (2004). Diet, nutrition and the prevention of cancer. Public Health Nutr. 7, 187-200.

Kile, B.T., Schulman, B.A., Alexander, W.S., Nicola, N.A., Martin, H.M., and Hilton, D.J. (2002). The SOCS box: a tale of destruction and degradation. Trends Biochem. Sci. *27*, 235-241.

Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C.W. (2002). Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene *285*, 1-24.

Kotenko, S.V., and Pestka, S. (2000). Jak-Stat signal transduction pathway through the eyes of cytokine class II receptor complexes. Oncogene *19*, 2557-2565.

Kubo, M., Hanada, T., and Yoshimura, A. (2003). Suppressors of cytokine signaling and immunity. Nat. Immunol. *4*, 1169-1176.

Lacronique, V., Boureux, A., Valle, V.D., Poirel, H., Quang, C.T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J., and Bernard, O.A. (1997). A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science *278*:1309.

Laird, A.D., and Cherrington, J.M. (2003). Small molecule tyrosine kinase inhibitors: clinical development of anticancer agents. Expert Opin. Investig. Drugs *12*, 51-64.

Larsen, L., and Ropke, C. (2002). Suppressors of cytokine signalling: SOCS. Apmis *110*, 833-844.

Lee, S.O., Lou, W., Hou, M., de Miguel, F., Gerber, L., and Gao, A.C. (2003). Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clin. Cancer Res. *9*, 370-376.

Leonard, W.J., and O'Shea, J.J. (1998). Jaks and STATs: biological implications. Annu. Rev. Immunol. *16*, 293-322.

Levy, D.E., and Darnell, J.E., Jr. (2002). Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. *3*, 651-662.

Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., and Shuai, K. (1998). Inhibition of Statl-mediated gene activation by PIAS1. Proc. Natl. Acad. Sci. USA *95*, 10626-10631.

Lou, W., Ni, Z., Dyer, K., Tweardy, D.J., and Gao, A.C. (2000). Interleukin-6 induces prostate cancer cell growth accompanied by activation of stat3 signaling pathway. Prostate *42*, 239-242.

Luo, C., and Laaja, P. (2004). Inhibitors of JAKs/STATs and the kinases: a possible new cluster of drugs. Drug Discov. Today *9*, 268-275.

Marine, J.C., McKay, C., Wang, D., Topham, D.J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., and Ihle, J.N. (1999). SOCS3 is essential in the regulation of fetal liver erythropoiesis. Cell *98*, 617-627.

May, T.S. (2003). Gleevec: tailoring to fit. Drug Discov. Today *8*, 188-189.

Metcalf, D., Alexander, W.S., Elefanty, A.G., Nicola, N.A., Hilton, D.J., Starr, R., Mifsud, S., and Di Rago, L. (1999). Aberrant hematopoiesis in mice with inactivation of the gene encoding SOCS-1. Leukemia *13*, 926-934.

Monni, R., Santos, S.C., Mauchauffe, M., Berger, R., Ghysdael, J., Gouilleux, F., Gisselbrecht, S., Bernard, O., and Penard-Lacronique, V. (2001). The TEL-Jak2 oncoprotein induces Socs1 expression and altered cytokine response in Ba/F3 cells. Oncogene *20*: 849-858.

Mora, L.B., Buettner, R., Seigne, J., Diaz, J., Ahmad, N., Garcia, R., Bowman, T., Falcone, R., Fairclough, R., Cantor, A., Muro-Cacho, C., Livingston, S., Karras, J., Pow-Sang, J., Jove, R. (2002). Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res. *62*, 6659-6666.

Naka, T., Nishimoto, N., and Kishimoto, T. (2002). The paradigm of IL-6: from basic science to medicine. Arthritis Res. *4 Suppl 3*, S233-242.

Okamoto, M., Lee, C., and Oyasu, R. (1997). Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. Cancer Res. *57*, 141-146.

Passmore, L.A., and Barford, D. (2004). Getting into position: the catalytic mechanisms of protein ubiquitylation. Biochem. J. *379*, 513-525.

Parganas, E., Wang, D., Stravopodis, D., Topham, D.J., Marine, J.C., Teglund, S., Vanin, E.F., Bodner, S., Colamonici, O.R., van Deursen, J.M., Grosveld, G., Ihle, J.N. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. Cell *93*, 385-395.

Peeters, P., Raynaud, S.D., Cools, J., Wlodarska, I., Grosgeorge, J., Philip, P., Monpoux, F., Van Rompaey, L., Baens, M., Van den Berghe, H., and Marynen, P. (1997). Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood 90:2535-2540.

Pestka, S., Kotenko, S.V., Muthukumaran, G., Izotova, L.S., Cook, J.R., and Garotta, G. (1997). The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor. Cytokine Growth Factor Rev. *8*, 189-206.

Pfitzenmaier, J., Vessella, R., Higano, C.S., Noteboom, J.L., Wallace, D., Jr., and Corey, E. (2003). Elevation of cytokine levels in cachectic patients with prostate carcinoma. Cancer *97*, 1211-1216.

Rane, S.G., and Reddy, E.P. (2000). Janus kinases: components of multiple signaling pathways. Oncogene *19*, 5662-5679.

Ritter, C.A., and Arteaga, C.L. (2003). The epidermal growth factor receptor-tyrosine kinase: a promising therapeutic target in solid tumors. Semin. Oncol. *30*, 3-11.

Roberts, A.W., Robb, L., Rakar, S., Hartley, L., Cluse, L., Nicola, N.A., Metcalf, D., Hilton, D.J., and Alexander, W.S. (2001). Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc. Natl. Acad. Sci. USA *98*, 9324-9329.

Robinson, D.R., Wu, Y.M., and Lin, S.F. (2000). The protein tyrosine kinase family of the human genome. Oncogene *19*, 5548-5557.

Rogers, R.S., Horvath, C.M., and Matunis, M.J. (2003). SUMO modification of STAT1 and its role in PIAS-mediated inhibition of gene activation. J. Biol. Chem. *278*, 30091-30097.

Rottapel, R., Ilangumaran, S., Neale, C., La Rose, J., Ho, J.M., Nguyen, M.H., Barber, D., Dubreuil, P., and de Sepulveda, P. (2002). The tumor suppressor activity of SOCS-1. Oncogene *21*, 4351-4362.

Sakamoto, H., Yasukawa, H., Masuhara, M., Tanimura, S., Sasaki, A., Yuge, K., Ohtsubo, M., Ohtsuka, A., Fujita, T., Ohta, T., Furukawa, Y., Iwase, S., Yamada, H., Yoshimura, A. (1998). A Janus kinase inhibitor, JAB, is an interferon-gamma-inducible gene and confers resistance to interferons. Blood *92*, 1668-1676.

Samuel, C.E. (2001). Antiviral actions of interferons. Clin. Microbiol. Rev. *14*, 778-809.

Schindler, C.W. (2002). Series introduction. JAK-STAT signaling in human disease. J. Clin. Invest. *109*, 1133-1137.

Schlessinger, J. (2003). Signal transduction. Autoinhibition control. Science *300*, 750-752.

Shawver, L.K., Slamon, D., and Ullrich, A. (2002). Smart drugs: tyrosine kinase inhibitors in cancer therapy. Cancer Cell *1*, 117-123.

Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. Nat. Rev. Immunol. *3*, 900-911.

Somani, A.K., Yuen, K., Xu, F., Zhang, J., Branch, D.R., and Siminovitch, K.A. (2001). The SH2 domain containing tyrosine phosphatase-1 down-regulates activation of Lyn and Lyn-induced tyrosine phosphorylation of the CD19 receptor in B cells. J. Biol. Chem. *276*, 1938-1944.

Smith, P.C., Hobisch, A., Lin, D.L., Culig, Z., and Keller, E.T. (2001). Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev. *12*, 33-40.

Starr, R., Metcalf, D., Elefanty, A.G., Brysha, M., Willson, T.A., Nicola, N.A., Hilton, D.J., and Alexander, W.S. (1998). Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc. Natl. Acad. Sci. USA *95*, 14395-14399.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annu. Rev. Biochem. *67*, 227-264.

Stone, K.R., Mickey, D.D., Wunderli, H., Mickey, G.H., and Paulson, D.F. (1978). Isolation of a human prostate carcinoma cell line (DU 145). Int. J. Cancer *21*, 274-281.

Szente, B.E., Weiner, I.J., Jablonsky, M.J., Krishna, N.R., Torres, B.A., and Johnson, H.M. (1996). Structural requirements for agonist activity of a murine interferon-gamma peptide. J. Interferon Cytokine Res. *16*, 813-817.

Szente, B.E., Subramaniam, P.S., and Johnson, H.M. (1995). Identification of IFN-gamma receptor binding sites for JAK2 and enhancement of binding by IFN-gamma and its C-terminal peptide IFN-gamma(95-133). J. Immunol. *155*, 5617-5622.

Tartaglia, M., Niemeyer, C.M., Shannon, K.M., and Loh, M.L. (2004). SHP-2 and myeloid malignancies. Curr. Opin. Hematol. *11*, 44-50.

Tsygankov, A.Y. (2003). Non-receptor protein tyrosine kinases. Front. Biosci. *8*, s595-635.

Traxler, P., Bold, G., Buchdunger, E., Caravatti, G., Furet, P., Manley, P., O'Reilly, T., Wood, J., and Zimmermann, J. (2001). Tyrosine kinase inhibitors: from rational design to clinical trials. Med. Res. Rev. *21*, 499-512.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D.J., and Silvennoinen, O. (2002). Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol. Cell Biol. *22*, 3316-3326.

Villain, M., Jackson, P.L., Manion, M.K., Dong, W.J., Su, Z., Fassina, G., Johnson, T.M., Sakai, T.T., Krishna, N.R., and Blalock, J.E. (2000). De novo design of peptides targeted to the EF hands of calmodulin. J. Biol. Chem. *275*, 2676-2685.

Vadlamudi, R.K., and Kumar, R. (2003). P21-activated kinases in human cancer. Cancer Metastasis Rev. *22*, 385-393.

Verma, A., Kambhampati, S., Parmar, S., and Platanias, L.C. (2003). Jak family of kinases in cancer. Cancer Metastasis Rev. *22*, 423-434.

Wells, A. (1999). EGF receptor. Int. J. Biochem. Cell Biol. *31*, 637-643.

Wu, D.W., Stark, K.C., Dunnington, D., Dillon, S.B., Yi, T., Jones, C., and Pelus, L.M. (2000). SH2-Containing protein tyrosine phosphatase-1 (SHP-1) association with Jak2 in UT-7/Epo cells. Blood Cells Mol. Dis. *26*, 15-24.

Xia, L., Wang, L., Chung, A.S., Ivanov, S.S., Ling, M.Y., Dragoi, A.M., Platt, A., Gilmer, T.M., Fu, X.Y., and Chin, Y.E. (2002). Identification of both positive and negative domains within the epidermal growth factor receptor COOH-terminal region for signal transducer and activator of transcription (STAT) activation. J. Biol. Chem. *277*, 30716-30723.

Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J.N., and Yoshimura, A. (1999). The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. Embo. J. *18*, 1309-1320.

Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000). Negative regulation of cytokine signaling pathways. Annu. Rev. Immunol. *18*, 143-164.

Yousefi, S., and Simon, H.U. (2003). SHP-1: a regulator of neutrophil apoptosis. Semin. Immunol. *15*, 195-199.

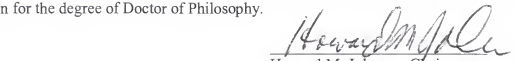
Zhang, P., Gao, W.Y., Turner, S., and Ducatman, B.S. (2003). Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect in vitro. Mol. Cancer *2*, 1.

Zhang, J., Somani, A.K., and Siminovitch, K.A. (2000). Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. Semin. Immunol. *12*, 361-378.

BIOGRAPHICAL SKETCH

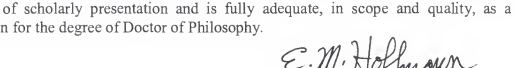
Lawrence O. Flowers was born in Richmond, Virginia, to Alvin and Gloria Flowers. He attended Harold M. Ratcliffe Elementary School, George H. Moody Middle School, and Henrico High School in Richmond, Virginia. Upon receiving his high school diploma he attended Virginia Commonwealth University (VCU) in Richmond, Virginia, where he majored in biology. After graduating from VCU with a Bachelor of Science degree in biology (*magna cum laude*) he matriculated at the University of Iowa (UI) in Iowa City, Iowa, where he majored in science education and biological sciences. After graduating with a Master of Science degree in science education and a Master of Science degree in biological sciences Lawrence began working on the Doctor of Philosophy degree at the University of Florida (UF) in Gainesville, Florida, in the Microbiology and Cell Science Department in the Fall of 2000.

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



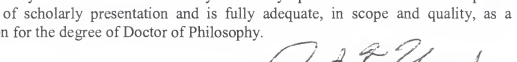
Howard M. Johnson, Chairman
Graduate Research Professor of
Microbiology and Cell Science

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



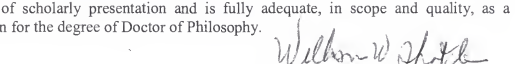
Edward M. Hoffmann
Professor Emeritus of Microbiology
and Cell Science

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



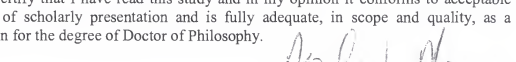
Janet K. Yamamoto
Professor of Pathobiology

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



William W. Thatcher
Graduate Research Professor of
Animal Sciences

I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Ayalew Mergia
Associate Professor of Pathobiology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for degree of Doctor of Philosophy.

August 2004

Dean, College of Agricultural and Life Sciences

Dean, Graduate School